University of Alberta

DIETARY REGULATION OF ARGININE SYNTHESIS IN THE NEONATAL PIGLET

by

DANA LEE WILKINSON ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Nutrition and Metabolism

Department of Agricultural, Food and Nutritional Science

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DEDICATION

For my husband Damian, who persevered along with me as I completed this degree, supporting me through his unending patience, assistance, and encouragement. Damian was never afraid to get his hands dirty in the lab or at the farm if I needed help, and his assistance both at home and at work has been invaluable to this project. He has truly taught me the meaning of unselfish living.

For my parents, growing up in their home was a blessing, and they have never stopped giving of themselves or their resources. You are so important in our lives and I thank you for teaching me the importance of a relationship with God.

> "Do not be anxious about anything, but in everything, by prayer and petition, with thanksgiving, present your requests to God. And the peace of God, which transcends all understanding, will guard your hearts and your minds in Christ Jesus." Philippians 5:6-7
>
> "For I know the plans I have for you, declares the Lord, plans to prosper you and not to harm you, plans to give you hope and a future." Jeremiah 29:11

ABSTRACT

Arginine is synthesized in the intestine of the neonate, however it is not known how endogenous arginine synthesis is regulated, and the degree to which proline and glutamate are used as precursors for arginine synthesis. Primed, constant intraportal and intragastric infusions of L-[U-^{14}C]proline and L-[3,4-^{3}H]glutamate, and intragastric L-[guanido-^{14}C]arginine were used to measure whole body and first pass intestinal arginine synthesis in 10 neonatal piglets fed arginine adequate (1.80g/kg/d) or deficient (0.20g/kg/d) diets for 5 days. Proline, but not glutamate, was an important precursor for arginine synthesis. Endogenous arginine synthesis from proline had obligatory (0.36g/kg/d), and maximal (0.68g/kg/d) levels. Whole body arginine synthesis from proline increased by 0.32g/kg/d ($p<0.01$) when dietary arginine was deficient, although first pass intestinal synthesis did not change. Arginine deficient animals demonstrated a trend toward lower nitric oxide production ($p<0.09$). Dietary arginine intake regulates whole body arginine synthesis from proline in the neonatal piglet.

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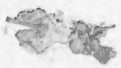

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1.0 INTRODUCTION

Prior to parturition, the placenta supplies a delicate balance of nutrients tailored to the needs of the developing fetus (Groh-Wargo et al. 1994). Premature birth interrupts the normal in utero development and nourishment of the fetus, leaving the fetus, now infant, only partially equipped for life ex utero. Early life for the premature infant is complicated by a host of nutritional challenges, such as limited nutrient reserves, immature absorption and metabolism, and high nutrient demands from rapid growth and development of tissues (Groh-Wargo et al. 1994). Nutritional interventions for these infants must promote optimal growth and development, without stressing poorly developed tissues and organs.

Because there are important ethical constraints to conducting research in the premature population, our knowledge of what constitutes optimal nutrition for these infants is limited. The development and validation of the neonatal piglet model has played a significant role in advancing this area of investigation (Ball et al. 1996) (see section 2.4). The neonatal piglet bears many important similarities to the human neonate, and in particular to the preterm neonate. Data from the piglet is most appropriately used as a model for these infants, and therefore comparisons between neonatal piglets and human neonates throughout this thesis are generally intended to refer to the preterm infant. Using this model, we have demonstrated that current amino acid solutions for the preterm neonate are unbalanced (Brunton et al. 1999), and this may have serious adverse consequences. Our overall goal is to improve the health and survival of preterm infants by determining the appropriate mixture of amino acids that should be provided to these infants during intravenous alimentation.

Although breast milk is considered to be the gold standard in nutrition for the term infant, the inherent variability of milk poses problems for the preterm infant. Breast milk frequently fails to meet the higher needs of preterm infants, imposed by a faster rate of growth and greater nutrient retention (Lucas 1993). Furthermore, the immaturity of the gut often precludes enteral feeding during the first days to weeks of life (Brunton et al. 2000). As a result, preterm infants are usually dependent upon the administration of special enteral and parenteral nutrition formulations. Compared to enteral feeding, total parenteral nutrition (TPN) is particularly unfavorable, because it limits gut growth and development, and causes gut atrophy. The absence of first pass small intestinal metabolism during TPN feeding is especially important with respect to protein nutrition, because there is extensive first pass intestinal metabolism of amino acids during normal oral feeding (Stoll et al. 1998).

Gut atrophy and the lack of first pass intestinal metabolism during intravenous nutrition severely limits arginine synthesis in the neonate, such that hyperammonemia rapidly develops in the absence of a dietary source of arginine (Brunton et al. 1999). The small intestine has been identified as the most important site of arginine synthesis in the neonate. Therefore, in the interest of defining the amino acid requirements of the neonate, there is a need to understand the regulation of intestinal arginine synthesis, whether dietary arginine intake plays a role in this regulation, and the relative importance of the various arginine precursors in these synthetic processes. This knowledge will help us to customize the delivery of arginine to preterm infants based on gut maturity and metabolic state, to distinguish between the needs of parenterally and enterally fed infants, and to establish a safe range of arginine intakes during enteral feeding.

2.0 LITERATURE REVIEW

2.1 AMINO ACID REQUIREMENTS OF THE INFANT
With particular reference to arginine

2.1.1 Classification of Amino Acids

There are 20 amino acids known to be incorporated into body protein, of these, 9 are classified as nutritionally indispensable for the neonate and the adult human (Table 2.1.1.). Endogenous synthesis of body proteins and of nitrogen containing molecules depends upon the provision of an adequate dietary supply of these indispensable amino acids, of non-essential nitrogen, and of non-protein energy (Groff et al. 1995). Although there is not a dietary need for what are termed the 'dispensable' amino acids (Table 2.1.1), they are essential components of protein, and provide a source of nitrogen for the synthesis of other amino acids and nitrogen containing compounds. The term 'conditionally indispensable' (Table 2.1.1) is used to describe those amino acids for which there are measurable limitations to their rate of synthesis (Reeds 2000). These limitations may arise from a reduced availability of precursor amino acids, from metabolic demands that exceed biosynthetic capabilities, or from dependence upon synthesis in a specific tissue, the metabolism of which may be affected by factors such as total parenteral nutrition (TPN) (Reeds 2000). Because the first limiting amino acid determines the rate of protein synthesis, a preformed source of the conditionally indispensable amino acids can be particularly important for neonates, who are in a critical stage of growth and development.

Table 2.1.1 Classification of amino acids and taurine as dispensable, indispensable, or conditionally indispensable for low birth weight (LBW) infants (Pencharz et al. 1996)

Dispensable	Indispensable	Conditionally Indispensable[a]
Alanine	Histidine	Arginine
Asparagine	Isoleucine	Cysteine
Aspartate	Leucine	Glutamine
Glutamate	Lysine	Glycine
Serine	Methionine	Proline
	Phenylalanine	Taurine[b]
	Threonine	Tyrosine
	Tryptophan	
	Valine	

[a]Insufficient evidence exists to determine whether these amino acids are dispensable or indispensable for the growth and development of LBW infants

[b]Taurine is not a component of protein

Arginine is a conditionally indispensable amino acid for infants. The gut is an important site of arginine synthesis in the neonate, and consequently, conditions of gut atrophy or dysfunction can impair synthesis (Brunton et al. 1999). Brunton et al. (1999) have demonstrated that arginine is indispensable in the diet of both parenterally and enterally fed neonates, regardless of gut health. Proline may also be indispensable for neonates, as suggested by indications of proline deficiency during enteral and parenteral administration of low proline or proline free diets to neonatal piglets (Samuels et al. 1989, Brunton et al. 1999).

Methionine is the precursor for the endogenous synthesis of cysteine. In the low birth weight (LBW) infant, however, reduced activity of hepatic cystathionase may limit cysteine production from methionine (Sturman et al. 1970). Similarly, although tyrosine can be produced via phenylalanine hydroxylation, a pre-formed source of tyrosine appears to be necessary for premature infants (Snyderman 1971). Finally, the LBW infant may have a reduced capacity for phenylalanine hydroxylation, although there is also evidence to the contrary (Pencharz et al. 1996).

The inclusion of glutamine in TPN formulations confers no apparent advantage to healthy neonates (House et al. 1994, Bertolo et al. 1999b), however, glutamine's role as an energy source for the small intestinal mucosa (Windmueller and Spaeth 1974) suggests that a pre-formed source may be required in some metabolic states (Lacey and Wilmore 1990). There is also speculation that glycine may be essential for LBW infants (Jackson et al. 1981), however further investigation is required. Although taurine is not a component of body protein, infants appear to require a dietary source of this amino acid as a result of low cysteine-sulfinic acid decarboxylase activity (Gaull et al. 1977).

2.1.2 Effect of Route of Feeding

The enteral administration of nutrients is normally regarded as superior to their parenteral administration. Complications such as reduced growth and functional development of the intestinal mucosa are commonly encountered when the route of feeding bypasses this important metabolic organ (Buchman et al. 1995, Groos et al. 1996). Because the gut catabolizes one third of dietary indispensable amino acids on first pass (Stoll et al. 1998), the absence or presence of first pass small intestinal metabolism has a substantial impact on amino acid requirements. Indeed, the requirement for many amino acids is lower during TPN feeding. Despite significant extraction of amino acids by the gut on first pass, Stoll et al. (1998) observed a net release of arginine, tyrosine, and alanine by the healthy gut of milk fed piglets. Our own research has confirmed the critical role of the gut for arginine synthesis in the neonate (Brunton et al. 1999, Bertolo et al. 2002). Indeed, rapid hyperammonemia developed in neonatal piglets fed arginine-free TPN (Brunton et al. 1999). Parenteral feeding induces gut atrophy and bypasses first pass gut metabolism, thereby compromising arginine synthesis. Therefore, the dietary requirement for arginine is higher during parenteral feeding, and may in fact approach the whole body arginine requirement because endogenous synthesis is limited.

2.1.3 Amino Acid Pattern in Milk

It appears logical to predict that the amino acid composition of a species' milk would correspond to the developmental and nutritional needs of the neonates of that species (Reeds et al. 2000). In reality, however, the amino acid composition of milk is imbalanced relative to needs for protein synthesis. For instance, glutamate, glutamine, proline, and leucine account for 40% of the total amino acid intake of milk fed neonates,

while arginine, alanine, and glycine are not supplied in adequate quantities (Table 2.1.2) (Reeds et al. 2000). These observations are equally true for both human and sow milk. Based on expected milk intakes (180mL/kg/d) and rates of protein deposition (2g/kg/d) in the human neonate, we have calculated that human milk provides only half of the daily arginine requirement for protein deposition in the neonate. That milk contains a relative excess of proline, glutamate, and glutamine may therefore be of great nutritional significance, because the neonate may utilize nitrogen and carbon skeletons from those amino acids present in excess, to synthesize those that are deficient (Reeds et al. 2000). Arginine and glycine are conditionally indispensable for the neonate, however, because their endogenous synthesis may be limited.

2.1.4 Amino Acid Pattern of TPN Formulations

Preterm infants are often unable to tolerate oral feeding in the first days to weeks of life because of the immaturity of their respiratory system and gastrointestinal tract (Brunton et al. 2000). Although not an ideal system of nutrient delivery, parenteral feeding must be the source of nutrition support for these infants. Parenteral solutions contain various quantities of dextrose, free amino acids, lipid, vitamins and minerals. Most of the components of parenteral nutrition solutions can be customized to the needs of the patient, however amino acids are added as ready-made commercial mixtures. The amino acid patterns of some of these formulations are based on reference proteins fed enterally such as human milk and egg, with modifications to achieve 'normal' plasma amino acid profiles (Ball et al. 1996). Despite different amino acid profiles, the ratio of indispensable to non-indispensable amino acids is similar among these solutions and human milk (Rassin 1986).

Table 2.1.2 Amino acid composition (g/100g protein) of piglet and human fetal tissue compared with human and swine milk (Pencharz et al. 1996)

Amino Acid	Piglet Tissue[a]	Swine Milk[b]	Human Fetal Tissue[c]	Human Milk[b]
Alanine	6.6	3.6	9.0	4.0
Arginine	6.9	4.4	7.7	3.6
Aspartate	8.0	7.8	13.0	8.6
Cysteine	1.5	1.6	n.d	2.0
Glutamate	13.8	20.8	11.8	19.0
Glycine	9.7	3.2	7.2	2.2
Histidine	2.5	2.4	2.6	2.3
Isoleucine	3.6	4.0	3.5	5.3
Leucine	6.9	8.9	7.5	10.4
Lysine	6.7	7.9	7.2	7.1
Methionine	1.6	2.2	2.0	1.6
Phenylalanine	3.7	4.3	4.1	3.7
Proline	6.7	11.7	8.4	9.5
Serine	4.2	5.1	4.1	6.1
Threonine	3.9	3.7	4.1	4.4
Tryptophan	n.d	1.4	n.d	1.9
Tyrosine	2.8	3.9	2.9	4.6
Valine	4.7	4.6	4.7	5.1

n.d: not determined

[a]Tissue of piglets 7 days of age (Aumaitre and Duee 1974)

[b]Davis et al. 1994

[c]Tissue of fetuses 2.9-3.4kg (Widdowson 1979)

The arginine concentration in commercial TPN solutions ranges from 4.7-12.3g/100g amino acids (Brunton et al. 2000). The administration of these solutions to the neonatal piglet would provide between 0.71 and 1.85g/kg/d of arginine, which is adequate only at the highest concentration. Indeed, a large number of studies have documented elevated plasma ammonia levels in TPN fed preterm infants when administered arginine at current TPN concentrations (Thomas et al. 1982, Seashore et al. 1982, Shohat et al. 1984). Batshaw and Brusilow (1978) have estimated that more than half of preterm infants experience elevated plasma ammonia levels, termed asymptomatic hyperammonemia. Furthermore, this transient asymptomatic hyperammonemia is accompanied by decreased levels of arginine and ornithine, and responds to oral administration of arginine (Batshaw et al. 1984).

2.2 ARGININE METABOLISM

2.2.1 General Characteristics and Metabolic Fates

Arginine is a basic, cationic amino acid that is the most abundant carrier of amino nitrogen, with 4 nitrogen atoms per molecule. It is a conditionally indispensable amino acid for infants and adults (Beaumier et al. 1996), and constitutes approximately 7% of the amino acids in piglet and fetal tissue (Pencharz et al. 1996). Arginine has a number of important of metabolic fates, of which urea synthesis is probably the most recognized. As both the immediate precursor for urea, and an allosteric activator for the synthesis of N-acetyl glutamate (NAG) (Beaumier et al. 1996), arginine plays a dual role in the synthesis, and regulation of urea production. Arginine is the only source of nitrogen for the formation of nitric oxide (NO) and creatine, and is a precursor for the synthesis of polyamines, which help to stabilize DNA during replication. At

supraphysiological doses, arginine is thymotropic, and is a stimulant of the immune system (Torosian 1994). It is a secretagogue for homones that control growth and metabolism (insulin, glucagon, growth hormone, and prolactin), while also being essential for the development of male sperm.

Arginine is one of the least toxic amino acids; doses up to 60g/d appear safe for humans (Barbul 1986). Deficiency is commonly manifest by hyperammonemia, due to the accumulation of excess ammonia in the plasma. Orotic aciduria is also an indication of arginine deficiency, and is evident in some disorders of urea cycle metabolism. This condition appears to result from an inadequate supply of ornithine relative to carbamyl phosphate production (Milner et al. 1975). Excess carbamyl phosphate is then shunted toward pyrimidine nucleotide synthesis, leading to the production of orotate.

Arginine, lysine, and ornithine are transported by the cationic amino acid transporter (CAT) family of carrier proteins. The four carriers that belong to this family are referred to as CAT-1, CAT-2A, CAT-2B, and CAT-3, and differ in their tissue distribution and substrate specificities (Hallemeesch et al. 2002). A number of "transport systems" have also been described for cationic amino acids. This term refers to transport activities measured in intact cells or membrane vesicles, and infers that specific transport activities may be mediated by a complex of different proteins, rather than by a single carrier protein (Closs and Graf 1999). System y^+ is a transport system that predominantly mediates the transport of L-arginine. It is expressed ubiquitously, with the exception of the liver. In the presence of Na^+, high concentrations of small neutral amino acids can interfere with y^+-mediated transport. Because arginine and lysine compete for uptake via this transport system, they can competitively inhibit uptake of each other, although this

competition may not be significant in the young pig (Edmonds and Baker 1987a). The expression pattern and transport activity of system y^+ is consistent with the CAT-1 family of carrier proteins, however it is not known whether they are one and the same (Closs and Graf 1999). Four other transport systems have been described for cationic amino acids, these differ from y^+ and from each other by their interaction with neutral amino acids.

2.2.2 Arginine and the Urea Cycle

Nitrogen is a potentially toxic waste product of amino acid catabolism generated by the liver, small intestine, kidney, and muscle (Brusilow and Horwich 1989). These waste nitrogen atoms must be incorporated into ammonium or aspartate and transferred to urea for excretion. This series of reactions is known as the urea cycle, and is depicted in Figure 2.2.1. Apart from disposing of waste nitrogen, the urea cycle also contains the reactions required for the de novo synthesis and degradation of arginine. Derangements in the cycle result in the accumulation of nitrogen atoms as ammonia and glutamine, and limit arginine synthesis (except in arginase deficiency where arginine accumulates due to decreased degradation).

Initiation and perpetuation of the urea cycle depends upon the action of several mitochondrial and cytosolic enzymes. Ornithine aminotransferase (OAT) and arginase are necessary for the conversion of Δ1-pyrroline-5-carboxylate (P5C) and arginine respectively, into ornithine, the first urea cycle substrate, which must then be transported into the mitochondria (Brusilow and Horwich 1989). Carbamyl phosphate synthase-1 (CPS-1) transforms ammonium and bicarbonate into carbamyl phosphate (CP). N-acetylglutamate (NAG, synthesized from glutamate and acetyl CoA) is an allosteric

Figure 2.2.1 Metabolic pathways associated with arginine

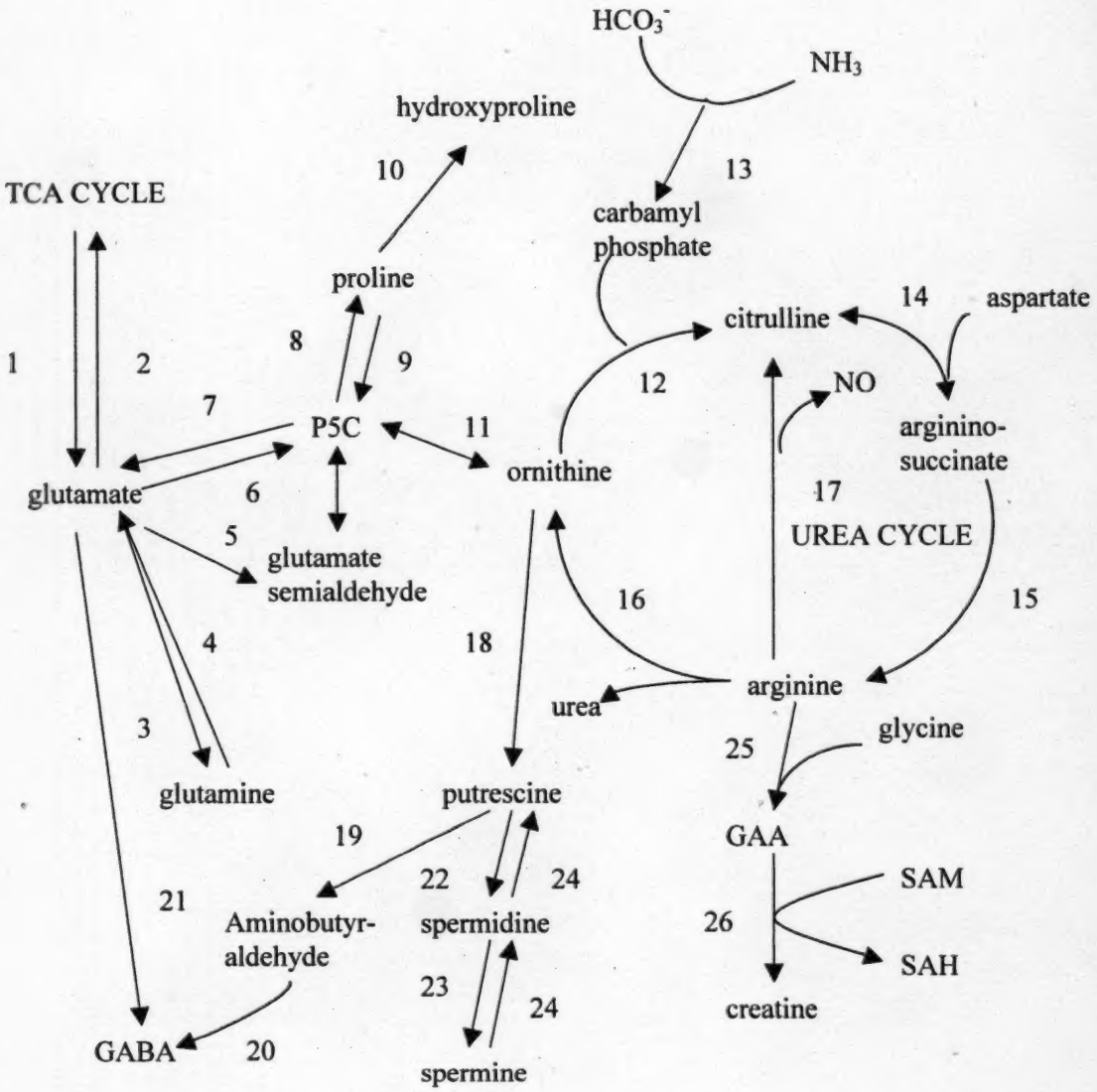


Table 2.2.1 List of enzymes associated with the metabolic pathways of arginine (Figure 2.2.1)

1	Glutamate Transaminase
2	Glutamate Dehydrogenase
3	Glutamine Synthase
4	Glutaminase
5	Glutamate Kinase and Reductase
6	Δ1-Pyrroline-5-Carboxylate (P5C) Synthase
7	Δ1-Pyrroline-5-Carboxylate (P5C) Dehydrogenase
8	Δ1-Pyrroline-5-Carboxylate (P5C) Reductase
9	Proline Oxidase
10	Prolyl-4-Hydroxylase
11	Ornithine-δ-Aminotransferase (OAT)
12	Ornithine Transcarbamylase (OTC)
13	Carbamyl Phosphate Synthase-1 (CPS-1)
14	Argininosuccinate Synthase (ASS)
15	Argininosuccinate Lyase (ASL)
16	Arginase
17	Nitric Oxide Synthase (NOS)
18	Ornithine Decarboxylase (ODC)
19	Diamine Oxidase (DAO)
20	Aldehyde Dehydrogenase
21	Glutamate Decarboxylase (GAD)
22	Spermidine Synthase
23	Spermine Synthase
24	Polyamine Oxidase (PAO)
25	L-arginine:glycine amidinotransferase
26	S-adenosyl-L-methionine:guanidinoacetate N-methyltransferase

cofactor for CPS-1, and is itself allosterically activated by arginine. Ornithine transcarbamylase (OTC) can then reversibly combine ornithine and CP to form citrulline in the mitochondria. Following diffusion into the cytosol, citrulline and aspartate become substrates for argininosuccinate synthase (ASS), producing argininosuccinate. Argininosuccinate lyase (ASL) cleaves argininosuccinate to fumarate and arginine. Finally, arginase hydrolyzes arginine to urea, thereby disposing of waste nitrogen, and reforming ornithine for a subsequent turn of the cycle.

The urea cycle does not appear to function at maximum capacity, and consequently, adjustments can be made to meet metabolic demands for urea synthesis (Visek 1979). An increased intake of dietary protein stimulates urea cycle enzyme activity in primates (Nuzum and Snodgrass 1971) and rats (Das and Waterlow 1974). In rats fed a high casein diet, alanine, glycine, and especially methionine were the amino acids responsible for increased activity of all 5 of the urea cycle enzymes (Snodgrass and Lin 1981). The urea cycle may also operate at higher rates in conditions where protein breakdown exceeds synthesis, such as in diabetes (McLean and Novello 1965) and starvation (Shih 1978). NAG is an important regulator of ureagenesis via its allosteric activation of CPS-1 for the synthesis of citrulline (Brusilow and Horwich 1989). Several lines of evidence indicate that ornithine may also be important in this regard, as high hepatic ornithine concentrations increase the capacity for hepatic ammoniagenesis (Kraus et al. 1976).

In the adult the complete urea cycle is expressed only in the liver (Morris 1992), although in the neonate it is also expressed in the intestine. Within the liver, there is a substantial compartmentation of arginine metabolism that permits the formation of

significant quantities of urea, without draining the cytosolic compartment of the arginine necessary for protein synthesis (Beaumier et al. 1996). Biologically, this organization is highly beneficial, as it obviates the need for major metabolic activity directed toward replenishment of arginine (Beaumier et al. 1996). A functional specialization of hepatocytes is also evident within the liver, known as 'metabolic zonation' (Jungermann and Katz 1989). Within this organization of enzymes, urea cycle enzymes are located periportally, including the potent cytoplasmic A1 isoenzyme of arginase known as 'liver type' (O'Sullivan et al. 1998). Arginine and ornithine catabolism through OAT occurs exclusively in the perivenous cells (O'Sullivan et al. 1998). The arginase present in this region may be the kidney type, mitochondrially associated, A2 arginase, although further characterization is required.

There is a similar compartmentation of urea cycle metabolism in the intestine, as urea cycle enzymes span 2 compartments in porcine enterocytes (Davis and Wu 1998). ASS, ASL, and arginase are located in the cytosol, and CPS-1, OTC, and arginase are found in the mitochondria. Two isoforms of arginase are present in the intestine, although further characterization is required. Compared to the liver, V_{max} values of urea cycle enzymes are much lower in enterocytes (except for OTC), and K_m values are much higher.

2.2.3 Arginine Synthesis and Regulation in the Adult

The activity of intestinal arginase is high in the adult, such that a large proportion of ingested arginine is hydrolysed to ornithine. Indeed in the fed adult human, 30-44% of an ingested arginine tracer is removed by the splanchnic region (Castillo et al. 1993b). Ornithine derived from dietary arginine, or alternatively from glutamine (Windmueller

and Spaeth 1980), is converted to citrulline via the action of intestinal CPS-1 and OTC. This citrulline, of which the intestine is the exclusive source (Windmueller and Spaeth 1981), is not further metabolized in the intestine, because ASS and ASL are present in trace quantities only (Jones 1985), and consequently a complete urea cycle does not operate in the adult intestine. Because CPS-1 activity in the small intestine is relatively low however, P5C is the major product of ornithine catabolism via the action of OAT (Wu 1998). OAT is a reversible enzyme whose activity favors the production of the metabolite (ornithine or P5C) present in the lowest concentration (Wu 1998). The high activity of small intestinal P5C reductase, coupled with low activity of P5C dehydrogenase, directs P5C towards the formation of proline. Thus, ornithine carbons are largely conserved for citrulline and proline synthesis (Wu 1998).

Endogenous arginine synthesis in the adult is dependent upon the synthesis and subsequent release of citrulline from the small intestine. The majority of circulating citrulline bypasses the liver (Windmueller and Spaeth 1981), and is instead metabolized in the kidney to arginine in stoichiometric amounts (Dhanakoti et al. 1990). Indeed, the availability of citrulline appears to be the limiting factor for renal arginine synthesis in the rat. Although the kidney lacks CPS-1 and OTC, ASS and ASL are present in the proximal tubules (Morris 1992), and thus intestinally derived citrulline is converted to arginine. Low renal arginase activity permits arginine release by the kidney for use by other tissues.

The metabolic flux of arginine in the adult is derived from 3 sources: dietary arginine that escapes splanchnic metabolism, endogenous synthesis via the cooperative actions of the intestine and kidney, and endogenous protein breakdown. The major route

of entry of arginine into the plasma in the adult is via endogenous protein breakdown (Beaumier et al. 1996), while only 11% of plasma arginine flux originates via conversion of citrulline to arginine (Castillo et al. 1996). Overall, the plasma arginine flux is low relative to what would be predicted on the basis of urea production (Castillo et al. 1993b). Indeed, the rate of urea production is 5-7 times greater than the plasma arginine flux, and the arginine formed via the urea cycle does not readily equilibrate and exchange with the liver and plasma free arginine pools (Beaumier et al. 1996). Only about 15% of plasma arginine turnover was associated with urea formation in adults (Castillo et al. 1996). These observations demonstrate a substantial compartmentation of arginine metabolism, and are the result of the near absence of the y^+ transport system in hepatocytes, which serves as a barrier between plasma and hepatic arginine pools (Closs and Graff 1999).

Isotopic investigations conducted in healthy adults fed arginine adequate or arginine free diets suggest that the rate of renal arginine synthesis is not upregulated in response to dietary arginine deficiency (Castillo et al. 1993a). Rather, when the dietary arginine intake is inadequate, homeostasis is achieved via altering the rate of arginine degradation, as verified by a 46% reduction in ornithine oxidation during intravenous infusion in healthy subjects fed arginine free diets (Castillo et al. 1994b). Similarly, adult burn patients who have a high rate of amino acid catabolism, do not have a higher rate of de novo arginine synthesis than healthy subjects (Yu et al. 1995a, b). Thus, renal synthesis does not appear to be upregulated in response to an increased need for arginine, and in conditions that increase amino acid oxidation, an exogenous source of arginine is necessary for the adult.

2.2.4 Arginine Synthesis and Regulation in the Neonate

Arginine metabolism differs in the neonate and the adult. Unlike in the adult, endogenous arginine synthesis is limited in healthy neonates, and thus an exogenous source of arginine is essential (Brunton et al. 1999). Because the activity of arginase is low in the neonatal intestine, the majority of dietary arginine enters the intestinal venous blood intact (Wu et al. 1996). Furthermore, the activities of ASS and ASL are high in neonatal enterocytes, and therefore, citrulline synthesized in the intestine is converted to arginine locally (Wu 1998). OAT may also be localized to the gut in the neonate (Bertolo et al. 2002). The suckling period in piglets has been described as a period of unusually high potential for OAT activity (Herzfeld and Raper 1976). The capacity for renal arginine synthesis appears to be limited by low renal ASL activity (Wu and Knabe 1995a), and thus the intestine is the major site of arginine synthesis in the neonate.

Marked changes in urea cycle and P5C amino acid metabolism occur over the course of early development, as the transition from predominantly intestinal, to predominantly renal arginine synthesis is made (Wu 1998). Indeed, in vitro measurements of piglet enterocytes indicate that the activities of all of the enzymes that synthesize arginine from proline and glutamine are highest at birth, and decline thereafter, with the exception of OAT, which peaks several days after birth (Wu and Knabe 1995a). The intestinal activity of the arginase enzyme is one of the key differences between the neonate and the adult, and is therefore particularly influential in determining the level and the location of endogenous arginine synthesis. The negligible activity of intestinal arginase at birth and during suckling, and subsequent induction at weaning profoundly affects the quantity and location of arginine synthesis (Flynn and Wu 1997). Weaning is associated with a number of enzymatic changes, inducing increases in

the activity of several arginine degrading enzymes (arginase, nitric oxide synthase (NOS), P5C reductase, and ornithine decarboxylase (ODC)), and in some arginine synthesizing enzymes (phosphate-dependent glutaminase (PDG), P5C synthase, and ASS) (Wu 1998). Similarly, in rat enterocytes, arginase activity begins to increase 3 weeks after birth when the transition is made from a milk-based to a normal diet, reaching adult levels 5 weeks after birth (Konarska and Tomaszewski 1975). In contrast, differences in intestinal arginase activities are much less pronounced when enterocytes from children up to 12 months old are compared to the adult. Arginase activity is in fact, only significantly greater in the duodenum of the adult (Konarska and Tomaszewski 1975). This may not be surprising, however, as infants are commonly introduced to solid foods at 6 to 9 months of age, and thus the induction of intestinal arginase activity may have already occurred in these infants. In summary, it appears that the transition from a milk-based diet is either a marker for, or an initiator of, the maturation of various arginine synthesizing and degrading enzymes in preparation for adult life.

The source of arginine flux in the neonate is identical to the adult, with the exception of endogenous arginine synthesis, which occurs in the gut. As in the adult, approximately 40% of an ingested arginine tracer is removed on first pass by the gut (Bertolo et al. 2002). The compartmentation of arginine metabolism has not been examined in the healthy neonate. Yu et al (1996) however, have noted a number of similarities between arginine metabolism in burned children (mean age 5.3y) and adults. Burned children exhibited little de novo arginine synthesis coupled with high rates of arginine degradation, as was also observed in the adult (Yu et al. 1995a, b).

Compartmentation between the hepatic urea cycle and plasma arginine pools was also evident.

The mechanisms governing the rate of endogenous arginine synthesis in the neonate have not been studied. Although arginine homeostasis in the adult is achieved via modulating the rate of arginine degradation, arginine metabolism in the neonate and the adult differs profoundly, and consequently this issue must be examined separately in the neonate. Intragastrically fed piglets become moderately hyperammonemic within hours of being fed an arginine free diet (Brunton et al. 1999), demonstrating the existence of an upper limit to the endogenous arginine synthetic capacity of the neonate. Furthermore, piglets fed suboptimal levels of arginine exhibit progressive improvements in indicators of arginine status (plasma ammonia, plasma arginine, muscle protein synthesis) with each incremental addition of arginine to the diet (Brunton et al. 2001, Wilkinson et al. 2002). This suggests that endogenous synthesis is not adequate to meet whole body arginine requirements. That there is a plateau over which these factors cease to improve indicates that over some range of intake, the diet, or a combination of diet + synthesis is able to satisfy the endogenous arginine requirement.

2.2.5 Proline as a Precursor for Arginine Synthesis in the Neonate

Proline, via P5C, is a precursor for the synthesis of glutamate and arginine, in a reaction catalyzed by proline oxidase, an enzyme that has been identified in intestinal tissue and cultured enterocytes from newborn, suckling, and weanling pigs (Wu 1997). The reverse pathway operates via P5C reductase, and therefore glutamate and arginine are also precursors for proline synthesis. In the neonate, these conversions are dependent upon gut metabolism (Murphy et al. 1996, Bertolo et al. 2002). Proline can also be

hydroxylated to hydroxyproline, a component of collagen. This is the principal route of hydroxyproline synthesis in mammals (Phang and Scriver 1989). There is no tRNA for the incorporation of hydroxyproline into protein, and thus the hydroxyproline found in various proteins occurs post-translationally. Hydroxyproline in its free form arises exclusively from the breakdown of dietary and endogenous sources of hydroxyproline-containing proteins (Phang and Scriver 1989).

Because the activities of the enzymes that synthesize arginine from proline are highest at birth, with the exception of OAT, proline is a particularly important precursor for arginine synthesis in the neonatal period (Wu and Knabe 1995a). In piglet enterocytes, the conversion rate of proline to arginine was greatest from age 0-7 days and dropped dramatically on day 7 (Wu 1997). From age 7 to 21 days the rate of conversion increased progressively, although the values remained much lower than in neonatal piglets. Furthermore, the vast majority of proline carbons, 80-90%, were recovered in ornithine, citrulline, and arginine.

The deficiency of arginine in sow and human milk suggests that the relative abundance of proline in these milks must make a significant contribution to endogenous arginine synthesis. The dietary intake of proline may regulate ornithine synthesis from proline (Murphy et al. 1996), as well as proline synthesis from arginine (Murch et al. 1996). Therefore, ample proline in the diet of suckling neonates may cause more proline to be diverted toward the pathways of arginine synthesis.

In vivo experiments have provided support for estimates of proline to arginine conversions obtained using isolated enterocytes. We have demonstrated that piglets intravenously fed arginine free, or arginine and proline free diets rapidly become

hyperammonemic (Brunton et al. 1999). Intragastric infusion of the same diets caused a less severe rise in plasma ammonia, although these animals would have eventually become hyperammonemic. In orally fed pigs, the rise in plasma ammonia was less severe when proline was included in the diet, demonstrating that proline can partially spare the arginine requirement during enteral, but not parenteral feeding. Using intragastric and intraportal infusions, we subsequently verified the importance of the gut for arginine synthesis from proline (Bertolo et al. 2002). According to our results, proline can partially spare the arginine requirement (Brunton et al. 1999), and vice versa (Murch et al. 1996). Therefore, arginine and proline should be considered co-indispensable (Bertolo et al. 2002).

2.2.6 Glutamate as a Precursor for Arginine Synthesis in the Neonate

Glutamate and serine are the only amino acids that can be synthesized de novo from a non-amino acid source of nitrogen, along with a carbon source (Reeds 2000). Endogenous synthesis has been demonstrated to make a quantitatively more important contribution to the flux of glutamate and serine than to the other dispensable amino acids. Indeed, dietary glutamate is virtually entirely metabolized upon first passage through the gut, and therefore the entire glutamate pool must derive from de novo synthesis via the reductive amination of α-ketoglutarate (Reeds et al. 1996). Thus, according to the strictest definition of the term, glutamate and serine alone can be labeled as dispensable amino acids, and are therefore the precursors of the other so called 'dispensable amino acids' (Reeds 2000).

A pivotal role in the transactions and economy of body nitrogen has been ascribed to glutamate (Young 2000). Indeed, glutamate acts as a donor of the nitrogen moiety to

the synthesis of all dispensable and conditionally indispensable amino acids (Reeds et al. 1996, Wu 1998), and comprises a significant portion of the tissue free amino acid pool (Young 2000). The initial steps of amino acid catabolism require glutamate for transamination and oxidative deamination reactions (Massey 1998). Transamination proceeds via the transfer of the alpha-amino N to alpha-ketoglutarate, forming glutamate (Massey 1998). Glutamate is then deaminated to yield alpha-ketoglutarate and ammonia. This transamination permits oxidative deamination of the indispensable amino acids, and acts as a means of converting many amino acids to TCA cycle intermediates. Together, glutamate and glutamine are critical to overall carbon and nitrogen metabolism, and link the carbon metabolism of carbohydrate and protein (Reeds et al. 2000).

P5C synthase can also catalyze the conversion of glutamate to P5C for subsequent entry into the urea cycle, and the pathways of arginine synthesis. The extent to which glutamate contributes to arginine synthesis has been difficult to ascertain, due to factors such as the large glutamate pool, nearly complete gut oxidation, and the many reactions required to synthesize arginine from glutamate. Investigations of isolated enterocytes from pre-weaning piglets have demonstrated that the conversion of glutamine to arginine is limited by low P5C synthase activity in the neonatal intestine (Wu et al. 1994). At birth, Blachier et al (1993) observed a very low level of arginine synthesis from glutamine, which was even lower in enterocytes from 2-8 day old animals. Although Blachier et al may have underestimated these levels of conversion, Wu and Knabe (1995a) subsequently showed that arginine synthesis from glutamine in 7 day old piglets was limited by reduced activities of PDG, P5C synthase, and CPS-1. Indeed, P5C synthase activity is high in newborn pigs, decreases within the first week after birth, and

then increases in 14 to 21 day old, and again in 29 to 58 day old animals (Wu et al. 1994). Plasma cortisol concentrations may mediate many developmental fluctuations in enzyme activities, enhancing the metabolism of glutamine and arginine, such that citrulline synthesis from glutamine (via induction of ASL and P5C synthase activities) is increased in weaned animals (Flynn and Wu 1997).

The suitability of glutamate as a precursor for arginine synthesis in the neonate has not been thoroughly investigated. In support of in vitro measurements of enzyme activities, Murphy et al (1996) did not detect any conversion of intragastrically administered glutamate to arginine in neonatal piglets. However, in milk fed piglets, 17% of mucosal arginine flux originated from intragastrically infused glutamate (Reeds et al. 2000). It is clear that the contribution of glutamate to arginine synthesis varies according to age, and therefore it must be distinctly characterized at various time points.

2.2.7 Other Metabolic Fates of Arginine

Creatine biosynthesis is a quantitatively important fate of arginine, described in Figure 2.2.1. The first step in this pathway occurs in the kidney, and involves the transfer of the amidino group of arginine to glycine by L-arginine:glycine amidinotransferase, yielding ornithine and guanidinoacetate (GAA) (Stead et al. 2001). GAA is then transported to the liver, where it is methylated by S-adenosylmethionine (SAM), forming creatine and S-adenosylhomocysteine (SAH) via the action of S-adenosyl-L-methionine:guanidinoacetate N-methyltransferase. Creatine is subsequently exported for transport to muscle, where it acts as a storage form of high energy phosphate for the rapid resynthesis of ATP (Beaumier et al. 1996). Creatine and creatine phosphate are converted to creatinine for excretion, resulting in the loss of arginine (Stead et al. 2001).

Under some conditions, the tissue arginine supply may become rate limiting for creatine synthesis, and consequently for muscle function (Beaumier et al. 1996). There is, however, feedback repression on L-arginine:glycine amidinotransferase at the pretranslational level, thereby preventing unnecessary consumption of arginine and methionine (Stead et al. 2001).

Arginine is a precursor for the synthesis of polyamines via its conversion to ornithine (Figure 2.2.1). Ornithine is a substrate for ornithine decarboxylase, the rate limiting enzyme in the formation of putrescine (Beaumier et al. 1996). The other 2 polyamines are formed from putrescine via the successive actions of spermidine synthase (forming spermidine) and spermine synthase (forming spermine) (Wu 1998). Similar to creatine synthesis, polyamine synthesis also requires methionine, in the form of decarboxylated 5-adenosylmethionine. Polyamines are low molecular weight compounds essential for cellular replication (Torosian 1994). They are particularly important for the proliferation and differentiation of rapidly dividing cells such as those in gastrointestinal mucosa, lymphocytes and fibrocytes, because they play a role in the synthesis of nucleic acids.

The conversion of arginine to NO is described in section 2.3.1.

2.2.8 Arginine Requirement Studies

There do not appear to be any published accounts of isotopic tracer studies in piglets or human infants designed to elucidate the arginine requirement. Consequently, the optimal level of arginine required by the neonate is not known. By convention, arginine is provided to piglets at levels set by the National Research Council (NRC). Human infants consume predetermined quantities of arginine through breast milk, and/or

through enteral and parenteral formulations designed to mimic reference proteins or to maintain a 'normal' plasma amino acid profile.

The NRC (1998) currently recommends 0.38g/kg/d of arginine for the neonatal piglet, a value extrapolated from studies in which graded levels of arginine were fed to weanling piglets (mean initial weight between 7.3 and 8.2kg) (Southern and Baker 1983). Maximal growth and feed efficiency were the main outcomes of interest in that study, and were maximized at a level of 0.48% of bioavailable dietary arginine. This level of intake was also associated with an increase in plasma urea nitrogen and a decrease in plasma ammonia compared with feeding lower levels of arginine. Urinary ammonia and orotate excretion were lower at this intake level, although urinary urea was unchanged. The authors concluded that the arginine requirement of the weanling piglet was 0.48% of the diet. This estimate was, however, based on relatively imprecise measurements, and should be reevaluated with more robust methodology such as radioactive tracers. Furthermore, there are marked changes in the activities of the urea cycle enzymes over the course of development (see section 2.2.4), and therefore the arginine requirement of the neonatal piglet cannot be extrapolated from older animals.

In 1959, Snyderman et al concluded that arginine was not required for the growing infant, based on growth and nitrogen balance data obtained from 3 infants who were not provided arginine for between 14 and 35 days. The frequent occurrence of arginine responsive hyperammonemia in preterm infants (Batshaw et al. 1984), however, suggests that arginine is indispensable for the neonate. We have furthermore established the indispensability of arginine during both enteral and parenteral feeding, although

proline can partially spare the dietary requirement for arginine during enteral feeding (Brunton et al. 1999).

In a recent study, we attempted to define the arginine requirement of the neonatal piglet (Brunton et al. 2001, Wilkinson et al. 2002). A range of arginine levels from 0.10 to 2.00g/kg/d were administered intragastrically or intravenously to neonatal piglets for 24 hours, and their ability to maintain normal plasma ammonia levels was monitored. Hyperammonemia was observed in piglets fed intragastric arginine up to 0.25g/kg/d (0.20g/kg/d of arginine was not included as a dietary treatment in this study), and in piglets fed intravenous arginine up to 0.45g/kg/d. In the intravenously fed piglets, muscle protein synthesis reached a plateau between 1.20 and 2.00g/kg/d of arginine. Plasma ammonia levels continued to decline linearly in these piglets up to 1.20g/kg/d, indicating that arginine was still limiting for urea synthesis below this intake. Plasma arginine concentrations reached a plateau between 0.90 and 2.00g/kg/d of arginine, at a concentration similar to the sow fed reference mean (Wykes et al. 1994). The absence of first pass intestinal metabolism in TPN fed piglets makes these animals an ideal model for elucidating the whole body arginine requirement. Therefore, these results in intravenously fed piglets suggested to us that the total metabolic demand for arginine was satisfied somewhere between dietary intakes of 1.20 and 2.00g/kg/d. The data in the intragastrically fed piglets is forthcoming.

Kinetic data have demonstrated that piglets fed 0.90g/kg/d of arginine synthesized 40% more arginine than was provided in the diet (Bertolo et al. 2002), yielding an estimated requirement of 1.70g/kg/d. From a theoretical standpoint, assuming that the neonatal piglet gains an average of 50g/d of protein, 6.9% of which is arginine (Pencharz

et al. 1996), a 2.5kg piglet would require 1.30g/kg/d of arginine for protein deposition alone. The additional arginine necessary for urea synthesis is difficult to estimate, as urea formation is believed to occur from intracellular arginine sources that are poorly or slowly equilibrated with the plasma pool (Castillo et al. 1996).

From these data we have estimated that 1.80g/kg/d of arginine is adequate to satisfy the total metabolic arginine requirement. There is no evidence to suggest that 1.80g/kg/d is an inadequate intake for the TPN fed piglet (Wilkinson et al. 2002), and indeed this level is 4.5 times greater than the current NRC estimated dietary requirement of 0.38g/kg/d (1998), and over 2.5 times the arginine intake from sow's milk (0.66g/kg/d) (Pencharz et al. 1996). These data underscore the fact that current feeding practices for the neonatal piglet and the human infant, particularly the TPN fed infant, are suboptimal.

The arginine requirement in the adult is entirely different from the neonate. Rose (1957) has previously shown that healthy young men do not require a dietary source of arginine for the maintenance of nitrogen equilibrium, and therefore classified arginine as a dispensable amino acid. Indeed, there is no evidence to suggest otherwise in healthy adults. Healthy adults fed arginine free diets for 6 days maintained arginine homeostasis via a reduction in the rate of arginine degradation, with no change in the level of endogenous synthesis (Castillo et al. 1993a, 1994a). Consequently in healthy adults, a dietary source of arginine is not necessary, however, in conditions that increase the oxidation of amino acids, such as burns and sepsis, an exogenous supply of arginine is necessary for the adult (Yu et al. 1995a, b).

2.3 ARGININE AND NITRIC OXIDE

2.3.1 Nitric Oxide

NO is the endothelium-derived relaxing factor (EDRF), a free radical whose nitrogen atom can exist in multiple oxidative states (Al-Ali and Howarth 1998). It is involved in the regulation of neurotransmission, vasodilation, and immunity (Bruins et al. 2002). NO is produced from arginine, and thus arginine is a common substrate for both urea and NO production (Figure 2.2.1). In a process termed the citrulline-NO cycle, NOS catalyzes the oxidation of arginine, releasing NO and citrulline in equimolar amounts (Mori and Gotoh 2000). Three isoforms of the NOS enzyme have been identified. Endothelial NOS (eNOS) and neuronal NOS (nNOS) are the constitutive (cNOS) isoforms of the NOS enzyme (Al-Ali and Howarth 1998). These isoforms produce NO in picomolar amounts in response to various physiological stimuli, generating a continuous supply of NO that acts as a transduction mechanism underlying critical physiological processes (Moncada et al. 1991), such as vasodilation (Galley et al. 1995) and the maintenance of normal endothelial cells (Loscalzo 2000). The inducible isoform (iNOS) of the NOS enzyme is part of the host defense reaction (Vallance 1999). It is a cytotoxic molecule induced by cytokines and endotoxins to produce nanomolar concentrations of NO for a shorter period of time (Al-Ali and Howarth 1998). This increased demand for NO is met via concomitant stimulation of ASS and sometimes of ASL to promote arginine synthesis (Mori and Gotoh 2000). Overproduction of NO by iNOS can contribute to tissue injury, hypotension, and myocardial failure (Bruins et al. 2002). Because arginine is a common substrate for NOS and arginase, induction and inhibition of arginase activity is an important endogenous means of regulating NO production by iNOS (Mori and Gotoh 2000).

2.3.2 Nitric Oxide and the Gut

NO is important in maintaining normal intestinal function. As the principal inhibitory neurotransmitter inducing gut smooth muscle relaxation, NO helps to regulate mucosal blood flow (Zamora et al. 1997). The diverse physiological and pathophysiological roles identified for NO as a neurotransmitter, antimicrobial agent, immunomodulator, intracellular signaling molecule, and vasodilator are evident in the gut (Tepperman and Whittle 2000). Through the activation of soluble guanylate cyclase, NO mediates changes in gut secretions, blood flow, electrolyte and water absorption, mucosal protection, and inflammation. Although normally released in small quantities by cNOS, overproduction of NO by iNOS can occur, resulting in cytotoxicity and gut inflammation.

Like many other mammalian cells, enterocytes utilize and also generate NO via the citrulline-NO cycle (Wu et al. 1996). During early development, NOS activity is highest in enterocytes from newborn and post-weaning pigs (Wu et al. 1996). Therefore, the gut simultaneously generates and consumes arginine for its own maintenance, albeit in very small quantities. Of the 1.2% of whole body arginine flux devoted to NO synthesis (Castillo et al. 1996), 16% of this conversion occurs on first pass by the gut in healthy subjects (Castillo et al. 1993c).

2.3.3 Therapeutic Modulation of Nitric Oxide Synthesis

Because NO is a mediator of biological function in almost all body systems (Al-Ali and Howarth 1998), NO production is an important therapeutic target for a number of pathological conditions. A loss of bioactive NO is central to the development of endothelial dysfunction, contributing to common disorders such as essential hypertension and atherothrombosis (Loscalzo 2000). Hyperproduction of NO, on the other hand, contributes to tissue injury, hypotension, and myocardial failure (Cook and Cattell 1996).

A number of potent pharmaceuticals target various aspects of NO synthesis and inactivation by increasing the availability of NO agonists, enzyme substrate and cofactors, or by limiting the generation of reactive oxygen species which can promote the oxidative inactivation of NO (Loscalzo 2000). The availability of arginine plays a key role in regulating NO production, and thus arginine and its metabolic enzymes are important therapeutic targets (Mori and Gotoh 2000). Mori and Gotoh (2000) have suggested that the inhibition of arginine transport or recycling, or the forced induction of arginase may prevent the overproduction of NO. Direct administration of arginine has also been shown to improve symptoms in conditions associated with impaired NO production (Loscalzo 2000).

2.3.4 Regulation of Nitric Oxide Production by Amino Acids

Although pharmaceuticals can often effectively treat disorders of NO production, dietary factors can be equally useful in the management of such conditions. As the only physiologically significant source of the nitrogen atom in NO, arginine is clearly the most important dietary regulator of NO synthesis (Morris 2000). Five factors are important in determining the availability of arginine for NO synthesis, they are: exogenous supply, endogenous release via protein degradation, arginine synthesis, catabolism, and transport (Morris 2000). The interplay of these factors, and their relative importance in various cell types, significantly complicates the relationship between arginine availability and NO production. Although NO production has been described as one of the four pathways "critical to maintain the body in condition", its synthesis may have little impact on overall arginine nutrition (Reeds 2000). In healthy men, the fraction of whole-body arginine flux used for NO formation was only 1.2% (Castillo et al. 1996).

Arginine has proven to be an important modulator of NO synthesis in a number of studies, although there is very little work in healthy individuals. The plasma arginine compartment appears to be a significant precursor pool for whole body NO formation (Castillo et al. 1996), and thus circulating arginine may determine the rate of NO production (Hallemeesch et al. 2002). In vitro work has demonstrated that extracellular arginine causes a dose dependent increase in NO production (Arnal et al. 1995). In young rats, dietary arginine deficiency impaired NO synthesis by cNOS and iNOS (Wu et al. 1999). In healthy adults, however, NO synthesis by cNOS was not affected by feeding of an arginine rich or an arginine free diet for 6 days (Castillo et al. 1995b). The apparent contradiction between these two results likely reflects the fact that arginine is dispensable in the diet of healthy adults, thereby limiting the impact of an arginine free diet (Morris 2000).

Elevated dietary arginine intake can exceed the capacity for arginine degradation (Morris 2000). This increased availability of arginine may explain why exogenous administration of arginine improves symptoms in individuals suffering from conditions associated with disordered NO production. The Km value of eNOS is, however, much lower than the intracellular arginine concentration, and thus the availability of arginine should not be limiting for NO synthesis. This phenomenon has been termed the "arginine paradox" (Wu and Meininger 2002).

In several studies of normal subjects, the administration of D-arginine was as effective as L-arginine in mediating vasodilation (Calver et al. 1991, Macallister et al. 1995, Rhodes et al. 1996). In many diseased animal models however, the effects of arginine are stereospecific (Girerd et al. 1990, Fineman et al. 1991, Eddahibi et al. 1992,

Shiraishi et al. 1997). By contrast, the effects of arginine were not stereospecific in diabetic and hypertensive subjects (MacAllister et al. 1995), while healthy subjects responded to L-arginine only (Imaizumi et al. 1992). Because D-arginine is not a substrate for NOS, it has been suggested that arginine's other actions may mediate some responses in normal subjects (Vallance 1999), which may explain the arginine paradox. These other actions include promoting the release of insulin, growth hormone, and prolactin (Calver et al. 1991, Macallister et al. 1995), arginine's anti-oxidant actions (Vallance 1999), or the high osmolality of amino acid infusates (Rhodes et al. 1996). This last hypothesis is consistent with the finding that the vasodilator response to either arginine or lysine in the resting forearm vasculature of normal man was similar (Rhodes et al. 1996). The reason why diseased animals often do not respond to D-arginine is not known, and such a response is not necessarily expected in humans. The data is clearly conflicting, and therefore it is not known whether the beneficial effects of arginine are the result of greater NO production, or whether they are mediated by arginine's other actions.

Arginine is not the only amino acid able to alter NO synthesis. In rats with a dietary protein deficiency, NO synthesis by cNOS was impaired, and could not be entirely attributed to a reduction in arginine availability (Wu et al. 1999). Interestingly, there is a well documented in vitro inhibition of NO formation by glutamine (Roth 1998) that may also exist in the rat (Houdijk et al. 1998). The administration of arginine is, however, able to reverse this inhibition in a dose dependent manner (Arnal et al. 1995). Wu et al (2001) have shown that glutamine metabolism to glucosamine-6-phosphate is necessary for this inhibition of NO synthesis by glutamine to occur in enterocytes. By

contrast, glutamate induces an increase in NO synthesis in brain and other nervous tissue (Atlante et al. 2001).

Citrulline and lysine appear to exert opposite effects on NO synthesis. Citrulline is recognized as an effective precursor for arginine synthesis, thus supporting NO synthesis (Morris 1999). Citrulline is also a neutral amino acid that does not compete with arginine for transport (Wu 2002). Lysine, however, does share the y^+ transport system with arginine, and thus NO production may be inhibited by high extracellular lysine concentrations (Hucks et al. 2000). There is direct and indirect evidence to suggest that high levels of homocysteine inhibit NO formation (Chao et al. 2000, Zhang et al. 2000).

A number of other dietary factors are able to influence NO synthesis, and these have been reviewed recently (Wu and Meininger 2002). Carbohydrates, fat, vitamins, minerals, glucosamine, phytoestrogens, ethanol and polyphenols may all regulate various aspects of NO synthesis. Although arginine may be crucial for NO synthesis, dietary regulation of NO synthesis is therefore not limited to arginine metabolism. A better understanding of NO production must consider the contribution of arginine, as well as other dietary components to the regulation of NO synthesis.

2.3.5 Arginine, and its Role in Nitric Oxide Mediated Pathologies of the Neonate

NO production in neonates may be adversely affected by the provision of suboptimal levels of arginine in commercial TPN solutions. Persistent pulmonary hypertension of the newborn (PPHN) is a NO related pathology that has attracted considerable attention in this regard. PPHN is the result of abnormalities in fetal lung development, such that the normal transition from a high resistance-low flow circulation

in the fetus to a low resistance-high flow circulation in the neonate does not occur, leading to hypoxia and severe distress for the neonate (Steinhorn and Russell 2000). The etiology of this condition is directly related to a defect in the NO signaling pathway, and thus many therapeutic interventions aim to manipulate this pathway (Steinhorn and Russell 2000). Inhaled NO significantly improves oxygenation in term infants with PPHN (Roberts et al. 1997), however it has potential toxicity in the lung (Beckman et al. 1990). As the precursor for NO synthesis, exogenous administration of arginine may therefore be beneficial for infants with PPHN.

Vosatka et al. (1994) have demonstrated that infants with PPHN have low plasma arginine concentrations, similar to those that can down-regulate NO synthesis in vitro. Intravenous infusion of arginine normalized plasma arginine levels in affected infants. Pearson et al (2001) verified the existence of low plasma arginine in infants with PPHN, and also documented diminished concentrations of NO metabolites in the plasma of these infants. Taken together, these results suggest that infants with PPHN have a reduced availability of arginine for conversion to NO.

Castillo et al. (1995) have published a more thorough comparison of whole body arginine metabolism during acute pulmonary vasoconstriction and convalescence. Arginine flux rates, urinary nitrate excretion, and the conversion rate of plasma arginine to NO were all lower during acute pulmonary hypertension. With increased provision of protein during convalescence, arginine flux and conversion to NO increased, while leucine flux was unchanged. These results support the hypothesis that arginine availability may become rate limiting for NO formation in infants with PPHN. Castillo et al. (1995) also suggest that a preferential shunting of arginine toward the formation of

ornithine and polyamines for pulmonary artery remodeling may deplete the arginine pool for conversion to NO.

The capacity for arginine uptake was impaired in isolated lungs of neonatal piglets chronically exposed to hypoxic conditions, compared to control animals (Fike et al. 2000). This impaired bioavailability of arginine may underlie the reduction in lung NO production observed in hypoxic lungs. The addition of arginine to the perfusate augmented the rate of accumulation of NO metabolites in the perfusate of hypoxic lungs only, supporting a positive role for arginine in the treatment of similar pathologies. Although it is not known whether direct infusion of arginine to human infants with PPHN increases NO production, such an infusion has been shown to improve pulmonary vascular resistance in these infants (McCaffrey et al. 1995). Furthermore, a recent case report documented that a combination of arginine and dipyridamole (a pharmaceutical that inhibits phosphodiesterase, the enzyme that hydrolyzes the smooth muscle relaxant cGMP) was successfully used to withdraw an infant with PPHN from inhaled NO (Saidy and Al-Alaiyan 2001). The potential efficacy of exogenous administration of arginine has been questioned by Kavvadia et al. (1999), who demonstrated that low plasma arginine levels are not necessarily present in all infants with PPHN. The preponderance of the evidence, however, suggests that the availability of arginine may be rate limiting for NO formation in PPHN, and thus exogenous administration of arginine to infants with PPHN may be highly beneficial in the treatment of this disorder.

Exogenous administration of arginine may also be of therapeutic benefit to infants suffering from necrotizing enterocolitis (NEC), the most common lethal intestinal injury in premature infants (Wu et al. 2000). Although the pathogenesis of NEC is not

completely understood, according to Kosloske (1990), NEC may result from the coincidence of at least 2 of the 4 pathological events: intestinal ischemia, bacterial colonization, excess protein substrate in the intestinal lumen and immunologic immaturity of the gut. Ischemia and reperfusion (I/R) injury have been considered as the major contributing factors to the development of NEC. However, in rats, I/R injury alone is not sufficient to induce NEC (Chan et al. 2002), and consequently Koloske's hypothesis may not fully describe the pathogenenic process. In young mice, hypoxia/reoxygenation (H/R) induces NEC, presumably via free oxygen radical damage (Akisu et al. 2002). NEC has been described as a cycle of inflammation and loss of the mucosal epithelial barrier (Insoft et al. 1996). Affected infants demonstrate feeding intolerance, bloody stools, intestinal inflammation, and pneumatosis intestinalis (the presence of air or gas in the gut) that may progress to gut perforation (Insoft et al. 1996).

NO is an important regulator of gastrointestinal blood flow, intestinal barrier function, and mucosal integrity (Amin et al. 2002). Arginine deficiency or immature NOS function may predispose premature infants to NEC because of the lack of NO-mediated gut protection (Amin et al. 2002). Preterm infants with NEC have low plasma arginine concentrations at the time of diagnosis (Zamora et al. 1997). Therefore, the administration of arginine, as the precursor for NO, may be therapeutic for infants suffering from NEC. Arginine infusion (0.6g/kg/h) in a neonatal piglet model of NEC significantly attenuates NEC-induced intestinal damage (Di Lorenzo et al. 1995). Similarly, enteral administration of arginine to young mice attenuates H/F-induced intestinal injury via increased endogenous NO production (Akisu et al. 2002). In a recent randomized, double-blind, placebo-controlled study of 152 premature infants, arginine

supplementation during the first 28 days of life significantly reduced the incidence of NEC (Amin et al. 2002). Plasma arginine concentrations were significantly lower in the infants who developed NEC compared to those who did not. Because there were no significant differences in plasma concentrations of total and indispensable amino acids, or in plasma glutamine concentrations in the neonates with NEC compared to those without, low plasma arginine concentrations were not the result of overall amino acid deficiency or increased catabolism, but were likely secondary to an increased metabolic demand for arginine or limited endogenous arginine synthesis (Amin et al. 2002). This study provides important data implicating arginine deficiency in the pathogenesis of neonatal disease.

2.4 THE PIGLET AS A MODEL FOR THE PRETERM HUMAN INFANT

Premature infants are a very fragile population (Ball et al. 1996). These infants are plagued by a variety of illnesses, often compounded by persistent infections, and difficulty recovering from surgery. It is not practically or ethically feasible to subject such a heterogenous population of infants to amino acid metabolism studies. The dietary manipulations necessary to gain important insight into the metabolism of a particular amino acid can have a significant impact on neonatal health and survival, particularly in light of the immaturity of the metabolic and immune systems of premature infants. However, this immaturity and fragility makes preterm infants dependent upon the provision of precise nutritional formulations to promote optimal growth and maturation. Development of such formulations must therefore rely heavily on studies of amino acid metabolism in an animal model. Clearly, such an animal model must bear a number of important similarities to the human infant, including similar pathways of amino acid

metabolism, and proportionately similar amino acid tissue composition and requirements. Practicality is also an issue, including cost, ease of procurement and housing, and appropriate size.

The biology of the pig is similar to the human in many respects such as renal morphology and physiology, skin morphology and physiology, dental characteristics, eye structure and visual acuity, and digestive anatomy and physiology (Pond and Houpt 1978). At many similar development points, hematological values for pigs resemble those of the human (Miller and Ullrey 1987). Both piglets and human infants experience a drop in body temperature at birth followed by a rise, have little thermal insulation, and demonstrate an increase in metabolic rate within a few days of parturition (Mount 1968). Furthermore, the body composition of the piglet resembles that of the preterm infant, particularly when the infant is born between 23 and 31 weeks of gestation (Shulman 1993).

According to Pond and Houpt (1978), the pig is the best nonprimate model for human nutrient requirements because of the physiological and anatomical similarity of the digestive tract of the pig and human. Comparison of each species pattern of nutrient requirements has revealed similarities during infancy, growth, reproduction, and lactation (Miller and Ullrey 1987). The piglet is particularly suitable for investigations of protein synthesis (Ball et al. 1996). Amino acid patterns in plasma, milk, and tissue (Table 2.1.2) are similar between piglets and human infants. Furthermore, the estimated requirements for indispensable amino acids are proportionately similar between the two species. It is nutritionally relevant that piglets are similar in size to human infants. This is important because body size affects the metabolic activity per unit weight and the organ distribution

for protein synthesis (Benevenga 1986). Consequently, data from the pig more closely matches the results expected in humans, than results scaled up from small laboratory animals.

Because the neonatal piglet grows at a faster rate than the human infant, amino acid requirements in the piglet are more closely tied to requirements for growth than for maintenance, while the reverse is true for the infant (Ball et al. 1996). This may lead to inaccuracies in requirement estimates for some amino acids, although the pattern of amino acid requirements during growth appear to be similar between the two species. The rapid growth rate of the piglet is beneficial in many ways, as it magnifies the effects of experimental manipulations (Shulman 1993), thereby facilitating detection of subtle changes in amino acid profiles that could escape notice in slower growing subjects (Ball et al. 1996). Furthermore, studies require less time than they would in some other slower growing models (Shulman 1993).

On a practical level, neonatal piglets can be obtained relatively easily and inexpensively. They represent a disease-free homogenous population, with known and repeatable genetics (Ball et al. 1996). They are relatively adaptable to rearing in an artificial environment, and their size facilitates handling while permitting collection of biological tissues and fluids in relatively large samples. Thus, both physiologically and practically, the neonatal piglet is the most appropriate non-primate model for the preterm human infant.

2.5 OBJECTIVES AND HYPOTHESIS

2.5.1 Objectives

In order to examine the dietary regulation of arginine synthesis in the neonate it

was first necessary to satisfy several preliminary objectives. In particular, the minimal dietary inclusion level for arginine that will provide a maximal synthetic challenge, but will prevent sustained hyperammonemia is not known, and must be defined. Data from our laboratory (Brunton et al. 2001, Bertolo et al. 2002, Wilkinson et al. 2002,) indicate that approximately 1.80g/kg/d of arginine should satisfy the total metabolic demand, however this needs to be tested. We also need to estimate the turnover time of the proline and glutamate pools in piglets fed these test diets so that we can accurately prime these pools during primed, constant infusion experiments. Using this information, we can then proceed to determine whether there is a maximal and a basal level of arginine synthesis, whether or not endogenous synthesis is regulated by dietary arginine, as well as the relative importance of proline and glutamate as precursors for arginine synthesis. Finally, we will test whether whole body NO synthesis is affected by the provision of adequate and deficient intakes of dietary arginine.

2.5.2 Hypotheses

1) The minimal dietary inclusion level for arginine will be 0.20g/kg/d of arginine.
2) The estimates for the turnover time of proline and glutamate in piglets fed adequate (1.80g/kg/d) or deficient (0.20g/kg/d) quantities of arginine will differ from those derived from piglets fed 1.00g/kg/d of arginine.
3) When arginine is provided at 0.20g/kg/d, arginine synthesis will increase, but will not be adequate to satisfy the total metabolic requirement for arginine.
4) When arginine is provided at a level of 1.80g/kg/d, arginine synthesis will continue to occur.

5) Proline, but not glutamate, will be an important precursor for arginine synthesis.

6) NO synthesis will be reduced when arginine is provided at 0.20g/kg/d.

3.0 DETERMINATION OF PROLINE AND GLUTAMATE KINETICS IN PIGLETS FED ARGININE ADEQUATE OR DEFICIENT DIETS

3.1 INTRODUCTION

Studies of amino acid kinetics are generally conducted via bolus or constant infusions of radiolabelled or stable isotopes. With a bolus infusion of isotope a number of parameters such as the half-life, turnover time, and pool size of the infused amino acid can be calculated, that cannot be determined from a constant infusion. Although the nature of the amino acid pool is better described by the kinetics of a bolus infusion, the relative simplicity of the constant infusion technique has made it the preferred method (Wolfe 1984). Unlike the bolus dose technique, the constant infusion method uses relatively simple mathematical equations, requires fewer blood samples, and is not subject to the same uncertainties regarding the nature and complexity of the volume of distribution of the substrate (Wolfe 1984). The kinetics of the constant infusion method are, however, only valid once an isotopic steady state is achieved (Bertolo PhD Thesis 1999). At this stage, the concentration of tracer entering the amino acid pool is equal to the concentration of tracer leaving the pool. To speed the achievement of a plateau in specific radioactivity (SRA), a priming dose of tracer is given prior to the constant infusion. This priming dose can be estimated via trial and error, or alternatively via a bolus infusion experiment.

A bolus infusion of isotope is administered to a pool system as a single dose, assumed to mix instantaneously with tracee, and to remain as such (Shipley and Clarke 1972). Over time, the reference amount of tracer in the pool declines. Ideally, a plot of this decline can be described by a single exponential function, which can be used to

derive various kinetic parameters (Wolfe 1992). The fraction of the contents of the pool that is being replaced per unit time is known as the rate constant for elimination, represented by k (Shipley and Clarke 1972). The value for k depends largely upon the input-output rate of tracee, as well as on the size of the pool of interest. Using k, an estimate of half-life and turnover time can be obtained. Half-life is the time required for the concentration or mass of tracer to decline by half at any point on the decay curve. Turnover time represents the time required for the total pool to move in and out of the system, and is equal to the prime/constant infusion (P/F) ratio. Because the SRA at the exact time of entry of tracer into the pool cannot practically be known, this value is inferred via extrapolation. The SRA at time=0 can then be used to obtain an estimate of the volume or mass of substance that constitutes the pool. Body pools exist in a dynamic equilibrium known as steady state, undergoing continual turnover with input equal to output. Although the single pool model assumes that the compartment of interest has no interchange with other body pools, in reality, such a pool does not exist in the animal body. However, if the injected tracer mixes quickly and thoroughly with other body compartments, this method can be useful, as it permits calculations of the parameters described above, that cannot be estimated from a constant infusion.

When the bolus injection technique is used, complex pools are better modelled using integral analysis. Here the decay of tracer is fitted to a curve, and the area under the curve is used to calculate the percent conversion of precursor to product, and the rate of appearance (Ra) of the substance of interest (Wolfe 1984). In theory, this approach should yield comparable values to those obtained with a constant infusion, however there may be a greater degree of variability associated with this type of analysis.

Unfortunately, with both the integral and the single pool analysis methods, the goodness of fit of the data to the exponential function is not predictive of accuracy. Because of the uncertainties inherent in these methods, the constant infusion technique is usually preferred. For this reason, the bolus infusion described in this chapter was followed by a constant infusion experiment.

We conducted a bolus infusion experiment to determine appropriate priming doses for [U-^{14}C]proline and [3,4-^{3}H]glutamate. A previous bolus experiment in our laboratory using [2,3-^{3}H]proline predicted a priming dose of 370kBq (10μCi) with a constant dose of 370kBq/kg/h (10μCi/kg/h) (P/F of 1.0) (Bertolo PhD Thesis 1999). Although we have never conducted a bolus experiment with glutamate, we have previously administered a priming dose of 185kBq (5μCi) prior to a 370kBq/kg/h (10μCi/kg/h) constant infusion (Murphy et al. 1996) with this isotope.

It was anticipated that the diets planned for administration in this experiment could alter tracer kinetics compared to previous observations. These diets contained what we hypothesized was an adequate and a deficient arginine intake for the neonatal piglet. We were particularly concerned about the potential metabolic consequences of feeding a diet with a deficient level of arginine for 5 days. This diet was formulated specifically to determine the lowest dietary arginine inclusion level that could prevent sustained hyperammonemia, and thus metabolic perturbations were expected. The purpose of this study was to validate the arginine content of these test diets, and to determine appropriate P/F ratios for proline and glutamate in piglets fed these diets.

The objectives of this experiment were: 1) To determine whether intragastrically fed, 1-3 day old piglets can maintain normal plasma ammonia levels on a diet containing

0.20g/kg/d of L-arginine; 2) To quantify the changes in P5C amino acid kinetics when arginine is deficient in the diet of newborn piglets; and 3) To determine P/F ratios for subsequent constant infusion experiments using [3,4-^{3}H]glutamate and [U-^{14}C]proline.

3.2 MATERIALS AND METHODS

3.2.1 Animals and Surgical Procedures

Six male Yorkshire piglets were obtained from the University of Alberta's swine research unit. All piglets were left with the sow until 1-2 days of age, and were then transferred to the metabolic unit for immediate surgical implantation of gastric and femoral vein catheters. All procedures used in this study received the approval of the University of Alberta Animal Care and Welfare Committee.

Piglets were initially anaesthetised with an intramuscular injection of acepromazine and ketamine hydrochloride, and subsequently maintained under a mixture of oxygen and halothane throughout surgery (day 0). Two custom-made silastic catheters were installed using aseptic technique (Wykes et al. 1993). A Stamm gastrostomy was performed in all piglets to install the gastric catheter, which was used for intragastric feeding and tracer infusion. The femoral vein catheter, used for blood sampling, was introduced into the left femoral vein, and was then advanced into the inferior vena cava just caudal to the heart. Catheters were tunnelled under the skin from the point of exit on the left side of the chest, to the points of entry into the blood vessels or stomach.

Immediately following surgery, piglets were given injections of buprenorphine hydrochloride (0.05mg) (Buprenex, Reckitt and Colman Pharmaceutical Inc, Richmond, VA), trimethoprim (10mg) and sulfadoxine (50mg) (Borgal, Hoechst Roussel Vet Canada Inc, Regina, SK), and gentamicin sulfate (5mg) (Garasol, Schering-Plough Animal

Health, Pointe-Claire, PQ). Gentamicin sulfate injections were continued for 3 days following surgery. Animals were housed individually in circular wire-mesh cages, allowing visual and audio contact with other piglets. Animals and cages were cleaned daily. Toys were provided for environmental enrichment. The room was lit from 0800 to 2000, and the temperature was maintained at 28°C, with supplemental heat provided by heat lamps.

3.2.2 Diets

The amino acid composition of the complete diet was similar to that of an elemental total parenteral nutrition (TPN) solution that is based on human milk protein (Vaminolact) (Table 3.2.1). The complete diet is designed to supply all the nutrients piglets require, when administered at targeted intakes of 15g amino acids/kg/d, 25g glucose/kg/d, and 11g lipid/kg/d, totalling 1.1MJ of metabolizable energy/kg/d. Protein, glucose, and lipid accounted for 23%, 39%, and 38% of energy intake respectively. According to our evidence (Brunton et al. 2001, Wilkinson et al. 2002), the concentration of arginine currently present in many commercial TPN solutions is insufficient to satisfy neonatal requirements for protein and urea synthesis. Consequently, the test diet that was designed to provide an adequate amount of arginine contained more arginine (12.78g/100g amino acids, Table 3.2.1) than the complete diet, providing 1.80g/kg/d of arginine for the neonatal piglet (Table 3.2.2). To ensure that diets were isonitrogenous, the concentrations of serine, alanine and glycine were adjusted in the diet formulation. For the arginine deficient (0.20g/kg/d, Table 3.2.2) test diet, the TPN base solution was free of arginine and serine. An excess of serine was added to the deficient diet to ensure

Table 3.2.1 Amino acid profile of the complete, arginine adequate (1.80g/kg/d) and arginine deficient (0.20g/kg/d) diets (g/100g amino acids)

Amino Acid	Complete (g/100g amino acids)	Adequate Arg (g/100g amino acids)	Deficient Arg (g/100g amino acids)
Alanine	10.59	5.27	9.93
Arginine	6.05	12.78	1.24
Aspartate	6.05	6.49	5.67
Cysteine	1.60	1.72	1.50
Glutamate	10.46	11.22	9.81
Glycine	2.72	1.46	2.55
Histidine	3.08	3.30	2.89
Isoleucine	4.59	4.92	4.30
Leucine	10.37	11.12	9.72
Lysine	8.25	8.84	7.73
Methionine	1.93	2.07	1.81
Phenylalanine	4.03	4.32	3.78
Proline	8.25	8.84	7.73
Serine	5.56	0.00	15.91
Taurine	0.45	0.48	0.42
Threonine	5.28	5.66	4.95
Tryptophan	2.11	2.26	1.98
Tyrosine	0.77	0.83	0.73
Valine	5.28	5.66	4.95
Glycyl-tyrosine	2.58	2.76	2.41

Table 3.2.2 Amino acid intake of piglets fed the complete, arginine adequate (1.80g/kg/d) and arginine deficient (0.20g/kg/d) diets (g/kg/d amino acids)

Amino Acid	NRC 1998[a] (g/kg/d)	Complete (g/kg/d)	Adequate Arg (g/kg/d)	Deficient Arg (g/kg/d)
Alanine	---	1.60	0.74	1.60
Arginine	0.38	0.91	1.80	0.20
Aspartate	---	0.91	0.91	0.91
Cysteine	0.30	0.24	0.24	0.24
Glutamate	---	1.58	1.58	1.58
Glycine	---	0.52	0.32	0.52
Histidine	0.30	0.47	0.47	0.47
Isoleucine	0.53	0.69	0.69	0.69
Leucine	0.95	1.57	1.57	1.57
Lysine	0.95	1.25	1.25	1.25
Methionine	0.25	0.29	0.29	0.29
Phenylalanine	0.58	0.61	0.61	0.61
Proline	---	1.25	1.25	1.25
Serine	---	0.84	0	2.56
Taurine	---	0.07	0.07	0.07
Threonine	0.63	0.80	0.80	0.80
Tryptophan	0.18	0.32	0.32	0.32
Tyrosine	0.30	0.39	0.39	0.39
Valine	0.65	0.80	0.80	0.80

[a]Requirement calculated from NRC 1998 (Table 10-6 amino acid requirements of pigs 3-5kg), assuming a 4kg piglet, a diet concentration of 3265kcal/kg ME, and an energy intake of 820kcal/d ME.

that both diets contained the same quantity of nitrogen. Prior to feeding, arginine and serine were dissolved in water and injected into sterile bags of TPN through a 0.22 μm filter, to provide 1.24g/100g amino acids and 15.91g/100g amino acids respectively.

Diets were first mixed and filtered into sterile bags. Just prior to diet infusion, vitamins (Multi-12K$_1$ Pediatric, Sabex, Boucherville, PQ) (Table 3.2.3), minerals (supplied at 200% NRC 1998 requirement) (Table 3.2.3), and lipid (Intralipid 20%, Pharmacia Inc., Mississauga, ON) were added to complete the TPN solution.

Immediately following surgery, the complete diet was infused intravenously via the femoral catheter at 50% of targeted intake until the morning of day 1. Diet infusion continued via the gastric catheter at 50% of full rate for 12h, 75% of full rate for 12h, and then at full rate (13.5mL/kg/h) until the morning of day 3.

On the morning of day 3, piglets were randomly assigned to receive either an arginine adequate (1.80g/kg/d), or an arginine deficient (0.20g/kg/d) diet for 5 days. The final group of 2 animals (1 per diet group) commenced test diet infusion beginning on the morning of day 2, continuing for 8 days.

3.2.3 Blood Sampling

Blood samples were collected from all piglets daily for the determination of nitric oxide (NO) and blood urea nitrogen (BUN) levels. In order to monitor plasma ammonia levels, blood was collected every 12 hours during test diet infusions. As described in section 3.2.4, additional blood samples were taken following bolus infusion of tracer.

3.2.4 Bolus Tracer Infusions

Table 3.2.3 Vitamin and mineral profile of diets

Vitamin	Concentration (/kg/d)
Ascorbic Acid	17.41mg
Vitamin A	500.48 IU
Vitamin D	87.04 IU
Thiamine (as hydrochloride)	0.26 mg
Riboflavin (as phosphate)	0.30 mg
Pyridoxine hydrochloride	0.22 mg
Niacinamide	3.70 mg
d-Panthenol	1.09 mg
Vitamin E (dl-alpha tocopheryl acetate)	1.52 IU
Vitamin K_1	0.04 mg
Biotin	4.35 µg
Folic Acid	30.46 µg
Vitamin B_{12} (cyanocobalamin)	0.22 µg
Mineral	**mg/kg/d**
Calcium	16.23
Phosphorus	12.51
Sodium	23.54
Chlorine	36.30
Magnesium	2.09
Potassium	15.79
Copper	0.86
Iodine	0.02
Iron	3.02
Manganese	0.67
Selenium	0.047
Zinc	15.58

Piglets were given a bolus intragastric dose (100uCi/kg) of each of L-[3,4-^{3}H]glutamate and L-[U-^{14}C]proline on the morning of day 6 or 7. Tracer solutions were prepared in 0.9% saline to achieve final concentrations of 100mCi/L. Immediately prior to isotope infusion, test diets were stopped temporarily to compensate for the approximate 5mL volume of isotope to be injected. Following injection, diet was immediately restarted, and continued for the duration of blood sampling.

In the first group of 2 piglets, blood was sampled over 8 hours. When the decay curve for glutamate was plotted, however, it was apparent that the tracer had not decayed during this interval. As a result, the period of blood sampling was extended to 24 hours for the next set of piglets. Because there was still no decay in the glutamate tracer over the 24 hour period, the final set of pigs was blood sampled over 99 hours. In all cases, blood sampling occurred every half hour for the first 3 hours, and hourly thereafter to 8 hours. In the second group of animals, blood was collected every other hour for an additional 8 hours, and once 8 hours later (24 hours post bolus infusion). In the final group, the additional blood samples were collected every 4 hours from 0800 to 2000 during the final 3 days of sampling. All samples were immediately centrifuged and frozen for subsequent analysis. Immediately following the last blood sample, piglets were euthanized via injection of sodium pentobarbital into the venous catheter.

3.2.5 Analytical Procedures

Plasma amino acids and the SRA of plasma glutamate, proline, and arginine were measured by reverse-phase HPLC using phenylisothiocyanate derivatives as previously described (House et al. 1997). Two internal standards, norleucine and L-[4,5-^{3}H]leucine (Amersham Pharmacia Biotech, St. Louis, MO), were added to each 300µl plasma

sample. Post-column radioactive derivatives were collected in 3 mL fractions, to which 15 mL of scintillant was added. Radioactive fractions were counted on a liquid scintillation counter using the dual isotope counting method for ^{3}H and ^{14}C.

Plasma ammonia concentrations were determined every 12 hours from the start of test diet infusions. The colorimetric assay used in the analysis was based on the amination of 2-oxoglutarate to glutamate, with simultaneous oxidation of NADPH (Sigma Procedure No. 171-UV, Sigma Diagnostics, St. Louis, MO). Plasma urea concentrations were measured daily using a spectrophotometric assay kit in which ammonia was liberated from urea by enzymatic hydrolysis (Sigma Chemical, St. Louis, MO). Plasma NO levels were determined via chemical reduction of nitrate to nitrite, followed by spectrophotometric analysis of total nitrite using Griess reagent (Oxis International Inc, Portland, OR).

3.2.6 Calculations

Amino acid concentrations were calculated as:

[Amino acid (μmol/L)] = (Amino acid peak area ÷ Norleucine peak area) * 0.10μmol norleucine added] ÷ volume of plasma in L

Plasma SRA's were calculated as follows:

SRA (dpm/μmol) = Amino acid radioactivity (dpm/200μL) ÷ [Amino acid (μmol/200μL)]

Both the "single-pool" and "integral analysis" methods as described by Wolfe (1992) were used to analyse plasma SRA data. For the "single-pool analysis" a monoexponential function was fitted to the data (Fig.P version 6.0c, Fig.P Software Corporation, Durham, NC):

$SRA_t = SRA_0 \times e^{-kt}$

where SRA_t (dpm/μmol) is the SRA at any time t, and k is the rate constant for elimination. These values were estimated by curve fitting software. Subsequent calculations were made to determine the following parameters:

Half-life (h) = ln2 ÷ k

Turnover time (h) = $t_{1/2}$ ÷ ln2

Pool size (μmol/kg) = tracer dose (dpm/kg) ÷ SRA_0 (dpm/μmol)

The SRA values that appeared to result from the initial incomplete mixing of tracer were "arbitrarily excluded" from the analysis (Wolfe 1992). Decay curves were then fitted from peak SRA values (these correspond to complete absorption of tracer) to 8h, 24h, or 99h.

The area under the curve was calculated for arginine and proline by integral analysis using a trapezoidal fit (Fig.P version 6.0c, Fig.P Software Corporation, Durham, NC). The % conversion between the infused tracer and the product of interest, arginine, was then calculated using the following equation:

% Conversion = (area under $curve_{amino\ acid\ product}$ ÷ area under $curve_{infused\ amino\ acid}$) x 100

3.2.7 Statistical Analyses

Statistical analyses were performed using the mixed model of SAS Version 8.3 (SAS Institute, Cary, NC). The dependent variables NO, ammonia, and BUN were analysed using a repeated measures analysis. The fixed effects were treatment (arginine adequate or deficient diet), while days and pig were the random effects. In all statistical models, the kenward-roger option was used to estimate denominator degrees of freedom.

The variance-covariance matrix chosen for each analysis was the best fit model based on the Schwarz's Bayesian Criterion. Least squares means were separated using the pdiff option when fixed effects were significant ($p<0.05$). A one-tailed test was used to compare plasma ammonia and BUN values between diet groups, because we expected these values to be significantly higher in arginine deficient compared to arginine adequate animals. Given the lack of comparable studies, a prediction could not be made regarding the result for the NO analysis, and hence a two-tailed test was employed.

All other comparisons between diet groups were performed using a two-tailed t-test (SAS Version 8.3, SAS Institute Inc, Cary, N.C.). Data were considered significantly different if $p<0.05$. Correlation coefficients (r^2) were generated by curve fitting software (Fig.P version 6.0c, Fig.P Software Corporation, Durham, N.C.).

3.3 RESULTS

The initiation of the arginine deficient diet did not precipitate a rise in plasma ammonia levels in 2 of 3 pigs receiving this diet. Although plasma ammonia levels did rise precipitously in one pig during the first night of test diet infusion, ammonia levels subsequently dropped. This animal appeared significantly more lethargic than the other animals that received the same diet, although he did not exhibit any of the other characteristic signs of hyperammonemia. Plasma ammonia levels were significantly greater in piglets receiving the deficient arginine diet throughout the period of test diet infusion ($p<0.04$, one-tailed test) (Figure 3.3.1). BUN levels were also significantly greater in these piglets ($p<0.02$, one-tailed test) (Figure 3.3.2). There were no significant trends in plasma NO concentrations by diet ($p>0.05$) (Figure 3.3.3).

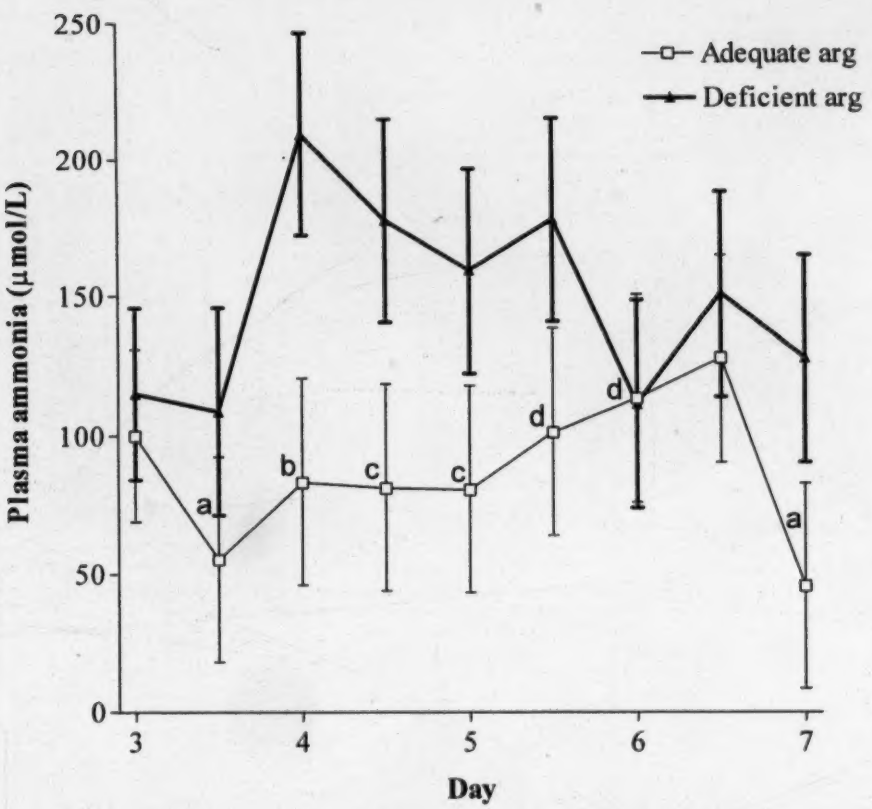


Figure 3.3.1 Mean plasma ammonia concentrations (µmol/L) of piglets fed an arginine adequate (1.80g/kg/d) or deficient (0.20g/kg/d) diet for 5 days. Plasma ammonia concentrations of piglets fed an arginine deficient diet were significantly higher than those of piglets fed an adequate diet ($p<0.04$, one-tailed test) as determined by a repeated measures analysis using the mixed model of SAS Version 8.3 (SAS Institute, Cary, NC). Data are means ± SEM, n=6. Least squares means were separated using the pdiff option when fixed effects were significant, superscripts are used to indicate the result of these comparisons.
[a]Point is different from arginine deficient on day 4am, 4pm, 5am, 5pm, 6pm.
[b]Point is different from arginine deficient on day 4am, 4pm, 5am.
[c]Point is different from arginine deficient on day 4am, 4pm, 5pm.
[d]Point is different from arginine deficient on day 4am.

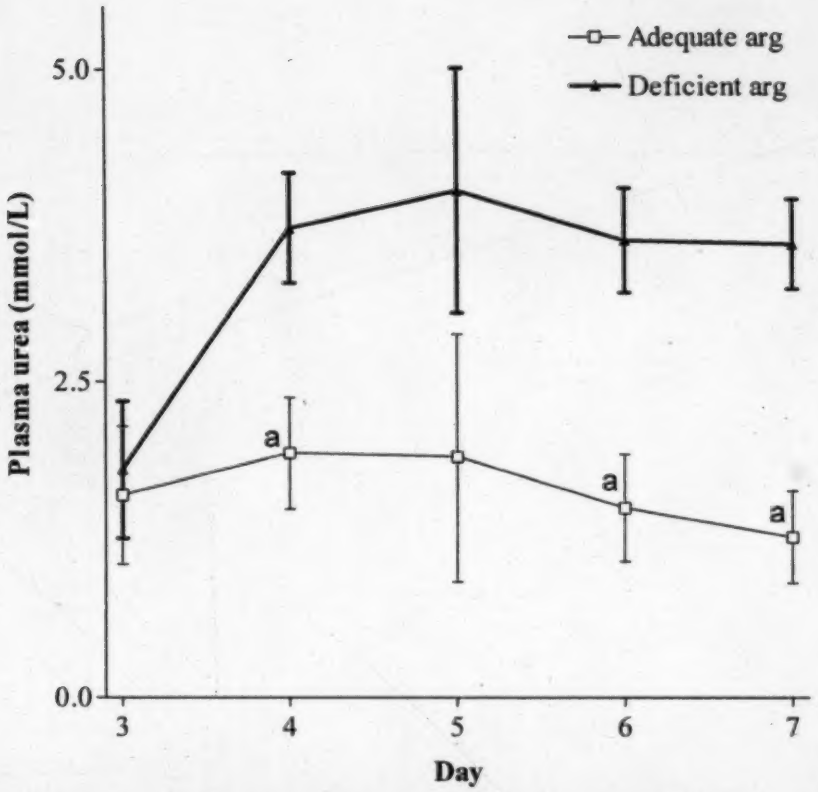


Figure 3.3.2 Mean plasma urea concentrations (mmol/L) of piglets fed an arginine adequate (1.80g/kg/d) or deficient (0.20g/kg/d) diet for 5 days. Plasma urea concentrations of piglets fed an arginine deficient diet were significantly higher than those of piglets fed an adequate diet ($p<0.02$, one-tailed test) as determined by a repeated measures analysis using the mixed model of SAS Version 8.3 (SAS Institute, Cary, NC). Data are means ± SEM, n=6.
Least squares means were separated using the pdiff option when fixed effects were significant, superscripts are used to indicate the result of these comparisons.
[a]Point is different from arginine deficient on day 4, 5, 6, 7.

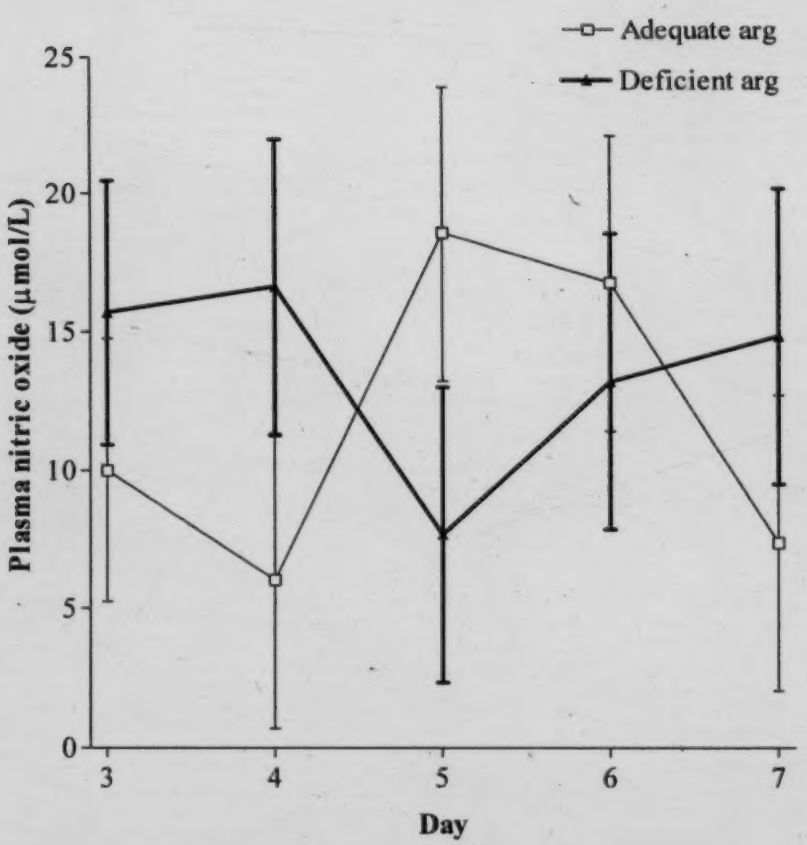


Figure 3.3.3 Mean plasma nitric oxide concentrations (µmol/L) of piglets fed an arginine adequate (1.80g/kg/d) or deficient (0.20g/kg/d) diet for 5 days. There was no significant effect of diet on plasma nitric oxide concentrations ($p>0.05$) as determined by a repeated measures analysis using the mixed model of SAS Version 8.3 (SAS Institute, Cary, NC). Data are means ± SEM, n=6.

Piglets given the arginine deficient diet had plasma arginine concentrations that were significantly lower than piglets given the arginine adequate diet ($p<0.01$) (Table 3.3.1). Plasma ornithine ($p<0.01$) was also significantly lower in piglets fed the deficient diet. Conversely, plasma glutamine concentrations were significantly higher in piglets receiving the arginine deficient diet ($p<0.01$).

Plasma concentrations of serine and glycine were significantly greater in piglets given the arginine deficient diet ($p<0.05$), while methionine ($p=0.01$) was significantly lower in piglets fed a deficient level of arginine (Table 3.3.1).

All animals remained healthy throughout the entire protocol, although piglets receiving the arginine deficient diet tended to become lethargic following test diet initiation. There was no significant difference between the two diet groups in body weight upon arrival, weight at initiation of test diets, or in total weight gain during complete and test diet infusions ($p>0.05$) (Table 3.3.2). The rates of body weight gain on all diets were not different from each other ($p>0.05$), and were within one standard deviation of the sow-fed reference mean (Wykes et al. 1993).

SRA data for the bolus infusion of [U-^{14}C]proline fit the monoexponential function (with residual = 0) very well. Typical decay curves for proline are presented in Figure 3.3.4. R^2 values for proline ranged from 0.96-1.00. Table 3.3.3 presents the kinetic parameters derived from the proline decay curves (half-life, turnover time (P/F), pool size), as well as plasma tracer concentrations. These parameters were not significantly affected by the level of arginine provided in the diet ($p>0.05$).

The percent conversions using the integral method from bolus infusions are presented in Table 3.3.3. In piglets receiving an arginine adequate diet, the percent

Table 3.3.1 Plasma amino acid concentrations (μmol/L) of neonatal piglets intragastrically fed adequate (1.80g/kg/d) or deficient (0.20g/kg/d) quantities of arginine for 5 days

Amino Acid	Adequate Arg	Deficient Arg	SEM	Sow-fed Reference Range[1]
		μmol/L		
Indispensable				
Histidine	54	62	11	47-121
Isoleucine	105	85	12	81-143
Leucine	**303**	**197**	31	102-175
Lysine	**571**	**594**	108	77-317
Methionine	**79**	**37**[a]	7	33-74
Phenylalanine	80	70	10	34-86
Threonine	**723**	408	124	214-500
Tryptophan	45	54	11	
Valine	259	223	22	175-318
Conditionally Indispensable				
Arginine	**380**	**22**[a]	43	50-267
Cysteine	5	8	34	
Glutamine	49	**217**[a]	22	
Glycine	**993**	**2119**[a]	295	450-976
Proline	674	651	71	304-890
Taurine	84	109	18	
Tyrosine	77	**84**	10	88-231
Other				
Alanine	605	710	93	413-1015
Aspartate	**11**	**4**	3	22-63
Citrulline	85	**69**	13	73-151
Glutamate	162	**67**	37	92-217
Hydroxyproline	127	97	17	
Ornithine	**339**	**88**[a]	32	79-195
Serine	192	**1231**[a]	146	137-442

n=6

[1]Data from Wykes et al (1994)

[a]$p<0.05$ compared to piglets fed adequate arginine diets

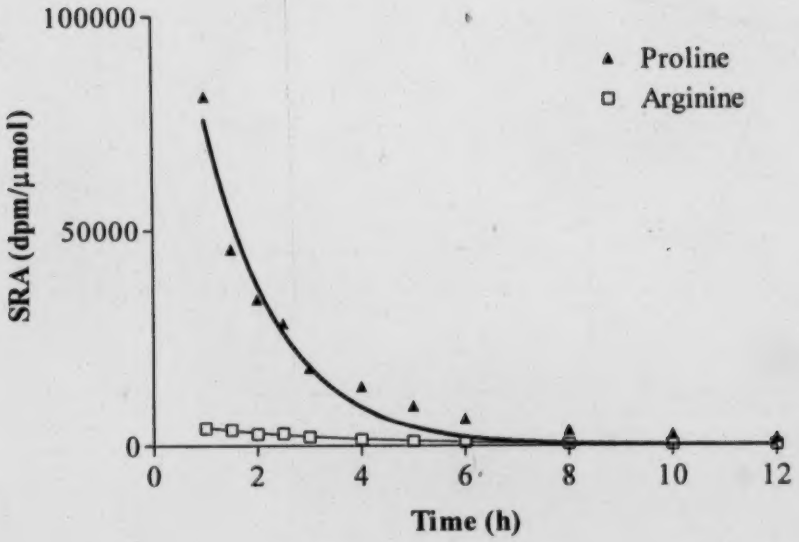
Bolded numbers indicate that these values were outside the sow-fed reference range

Table 3.3.2 Performance of neonatal piglets intragastrically fed adequate (1.80g/kg/d) or deficient (0.20g/kg/d) quantities of arginine for 5 days

	Adequate Arg	Deficient Arg	SEM
Weight (study d0) (kg)	1.70	1.65	0.08
Weight Gain Complete Diet (d0-3) (kg)	0.22	0.26	0.07
Rate of Weight Gain Complete Diet (d0-3) (g/kg/d)	70	89	9
Weight at Initiation of Test Diets (d3) (kg)	1.92	1.92	0.13
Weight Gain Test Diet (d3-7) (kg)	0.97	0.93	0.20
Rate of Weight Gain Test Diet (d3-7) (g/kg/d)	74	74	2

n=6

Data are not significantly different ($p>0.05$)



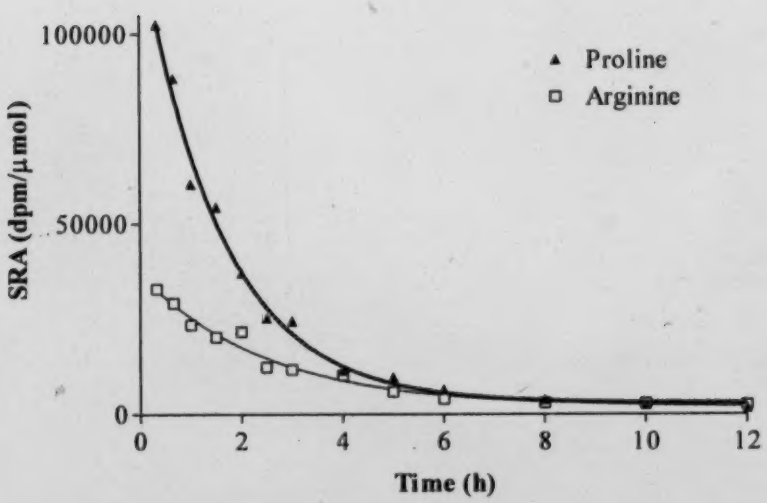


Figure 3.3.4 Typical plasma specific radioactivity decay curves after single bolus injection of intragastric [U-^{14}C]proline in piglets intragastrically fed an arginine adequate (1.80g/kg/d) (top) or deficient (0.20g/kg/d) (bottom) diet for 5 days. Curves were generated by data fitting software (Fig.P version 6.0c, Fig.P Software Corporation, Durham, NC) and represent fits for the monoexponential function $SRA_t = SRA_0 \times e^{-kt}$.

Table 3.3.3 Plasma proline kinetics derived from a bolus infusion of [U-^{14}C]proline in piglets intragastrically fed adequate (1.80g/kg/d) or deficient (0.20g/kg/d) quantities of arginine for 5 days

	Adequate Arg	Deficient Arg	SEM
Curve Fit (r^2)	0.99	0.98	0.01
Half-life (h)	1.22	1.47	0.26
Turnover Time (P/F)	1.76	2.12	0.37
Pool Size (μmol/kg)	1491	1972	370
Plasma Concentration (μmol/L)	674	651	71
% Conversion of Proline to Arginine	11	53[a]	4

n=6

[a]$p<0.01$ compared to piglets fed adequate arginine diets

conversion of proline to arginine was 11%, whereas in piglets fed a diet deficient in arginine, this conversion was 53%. These values differed significantly ($p=0.02$).

SRA data from the bolus infusion of [3,4-^{3}H]glutamate did not fit the monoexponential function very well (Figure 3.3.5). R^2 values were 0.01, 0.35, and 0.82 for the individual adequate arginine pigs, and 0.04, <0.01, and <0.01 for the deficient arginine pigs. Although the duration of blood sampling was extended from 8h to 24h, and finally to 99h, the concentration of radiolabelled glutamate in the plasma did not decay over time in the vast majority of cases. Figure 3.3.5 depicts the only instance where there was some decay observed in [3,4-^{3}H]glutamate, however it is evident that the SRA of the tracer remained static, and relatively high over the final 2.5 days of blood sampling. Because the data did not fit the monoexponential function, a value for k, the rate constant for elimination, could not be determined. Consequently, kinetic parameters for glutamate could not be calculated, and a P/F ratio was not determined. There was no apparent conversion of [3,4-^{3}H]glutamate to arginine, based on the observation that arginine radioactivity in the tritium channel did not differ from background at any time period on either diet.

3.4 DISCUSSION

An important objective of this work was to determine if piglets could maintain normal plasma ammonia levels on a diet containing 0.20g/kg/d (requirement 0.38g/kg/d, NRC 1998) of arginine. Plasma ammonia levels are an acutely sensitive measure of arginine status in healthy neonates (Brunton et al. 1999). The level of arginine included in the deficient diet was selected based on the results of a previous study, in which a range of arginine levels from 0.10 to 2.00g/kg/d were administered intragastrically to

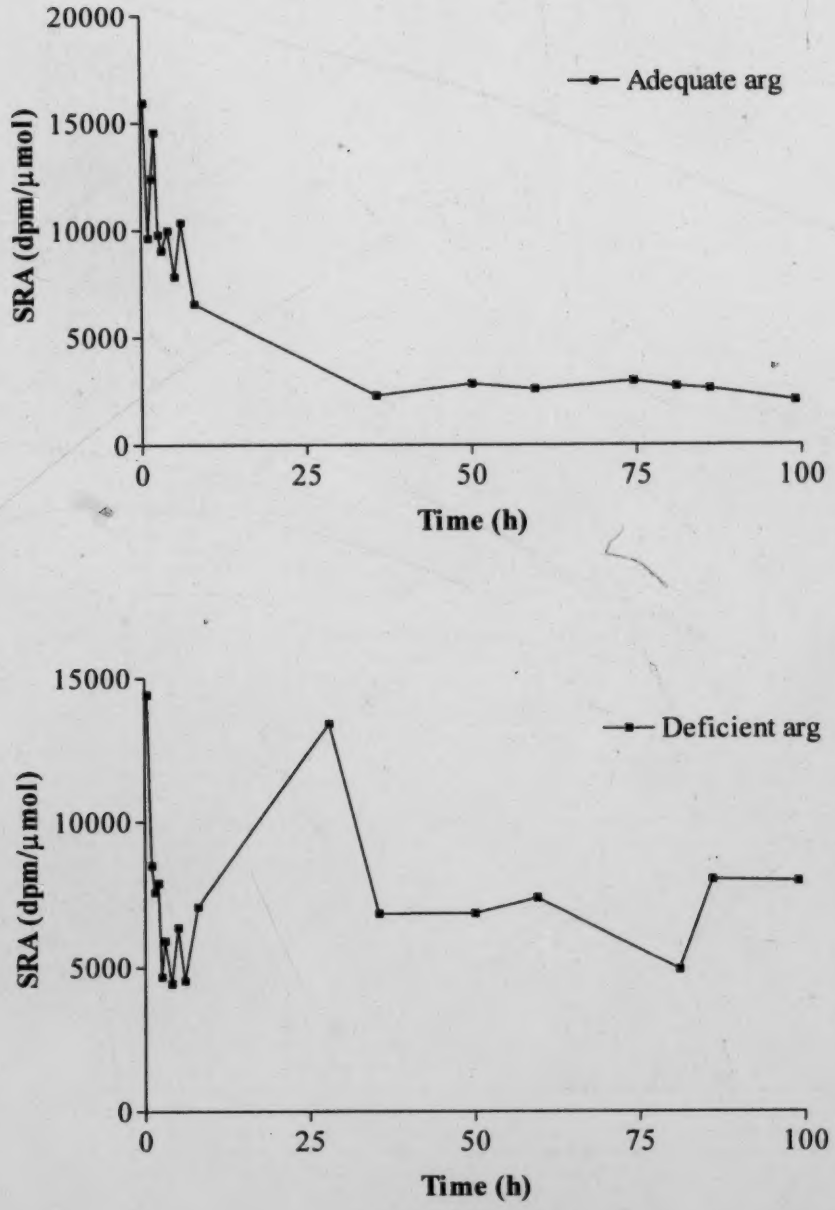


Figure 3.3.5 Typical plasma specific radioactivity decay curves after single bolus injection of intragastric [3,4-^{3}H]glutamate in piglets intragastrically fed an arginine adequate (1.80g/kg/d) or deficient (0.20g/kg/d) diet for 5 days. Curves were generated by data fitting software (Fig.P version 6.0c, Fig.P Software Corporation, Durham, NC) and represent fits for the monoexponential function $SRA_t = SRA_0 \times e^{-kt}$.

neonatal piglets for 24 hours, and their ability to maintain normal plasma ammonia levels was monitored (Brunton et al. unpublished). In that study, piglets receiving 0.15g/kg/d of arginine developed severe hyperammonemia after 8 hours, and were subsequently able to maintain moderate ammonia levels for the remaining 16 hours of the study. Animals fed 0.25g/kg/d of arginine became moderately hyperammonemic after 8 hours, with a return to normal levels over the next 16 hours. A dietary arginine level in between these two levels was chosen to provide the lowest possible arginine inclusion level that could prevent hyperammonemia. The metabolic consequences of sustained hyperammonemia are incompatible with survival, and thus we wanted to avoid such a state. The transient hyperammonemia observed in our animals, although not a desirable outcome, helped to establish that 0.20g/kg/d of arginine was a truly deficient intake. The piglets fed this diet had higher plasma ammonia levels than piglets fed an adequate quantity of arginine, however, they maintained sufficient urea cycle function to support moderate ammonia levels.

Our second objective was to examine changes in P5C amino acid kinetics induced by variations in the dietary level of arginine. We hypothesized that an arginine intake of 1.80g/kg/d would satisfy the total metabolic demand for arginine, and used this as the adequate intake level. In a recent study in our laboratory, muscle protein synthesis reached a plateau when neonatal piglets were intravenously fed between 1.20 and 2.00g/kg/d of arginine (Brunton et al. 2001, Wilkinson et al. 2002). Plasma ammonia levels continued to decline linearly in these piglets up to 1.20g/kg/d, indicating that arginine was still limiting for urea synthesis below this intake. Plasma arginine concentrations reached a plateau between 0.90 and 2.00g/kg/d of arginine, at a

concentration similar to the sow fed reference mean (Wykes et al. 1994). These results suggested to us that the total metabolic demand for arginine was satisfied somewhere between dietary intakes of 1.20 and 2.00g/kg/d. Kinetic data from another study demonstrated that piglets fed 0.90g/kg/d of arginine synthesized 40% more arginine than was provided in the diet (Bertolo et al. 2002). From a theoretical standpoint, assuming that the neonatal piglet gains an average of 50g/d of protein, 6.9% of which is arginine (Pencharz et al. 1996), a 2.5kg piglet would require 1.30g/kg/d of arginine for protein deposition alone. The additional arginine necessary for urea synthesis is difficult to estimate, because urea formation is believed to occur from intracellular arginine sources that are poorly or slowly equilibrated with the plasma pool (Castillo et al. 1996).

Our evidence suggests that 1.80g/kg/d of arginine provided an adequate quantity of arginine for the neonatal piglet. Plasma arginine concentrations in sow-fed piglets reflect the low arginine content of sow's milk relative to requirements for protein deposition (Pencharz et al. 1996). However in this study, piglets fed an adequate quantity of arginine had plasma arginine concentrations 40% greater than the highest values observed in sow-fed animals (Wykes et al. 1994). Furthermore, the animals receiving this diet appeared healthy, gained weight at expected rates, and maintained normal plasma ammonia levels. Plasma glutamine concentrations were significantly lower than those measured in piglets fed the deficient diet ($p<0.01$), because alternate ammonia disposal mechanisms were not required. Plasma methionine was significantly higher in pigs fed the higher arginine diet, although cysteine and cystathione concentrations were unaffected. The reason for such an increase is not known, however it may reflect an increase in remethylation from homocysteine. There is a recognized relationship between

this pathway and arginine metabolism, because high homocysteine decreases NO synthesis (Zhang 2000). Furthermore, methionine plays an important role in arginine metabolism via its donation of methyl groups during creatine and polyamine synthesis from arginine. The quantitative significance of this relationship should not be underestimated. Creatine synthesis has been estimated to consume approximately 70% of all physiologically labile methyl groups, more than all other methylation reactions combined (Wyss and Wallimann 1994). This pathway may also contribute to the ability of arginine plus glycine to alleviate the toxic effects of excess methionine in rats (Brown and Allison 1948), while worsening the growth depression of chicks fed diets limiting in methionine (Keshavarz and Fuller 1971). These findings suggest that there may be a previously underappreciated relationship between these two amino acids.

When the total metabolic demand for arginine is satisfied by the diet, arginine synthesis may no longer occur. Based on this assumption, if 1.80g/kg/d is truly an adequate intake of arginine, no radioactive label would be recovered from proline and glutamate in arginine. However, it is also possible that there is a basal level of arginine synthesis that occurs regardless of the dietary inclusion level of arginine. Our results demonstrate that arginine synthesis still occurs when the diet contains 1.80g/kg/d of arginine. Because this, and previous data (Brunton, et al. 2001, Wilkinson et al. 2002) leads us to believe that 1.80g/kg/d of arginine is adequate to satisfy the total metabolic demand for arginine, the synthesis that continues to occur must represent a basal level of obligatory arginine synthesis. Furthermore, although arginine flux was not measured in this study, our conversion data suggest that arginine synthesis from proline was downregulated in response to a dietary sufficiency of arginine.

Although piglets were successfully maintained on a diet deficient in arginine for 5 days, our results indicate that they were not able to synthesize sufficient arginine to meet their total metabolic requirements. Piglets fed the deficient diet had significantly lower plasma arginine concentrations than piglets fed an adequate level of arginine ($p<0.01$), which is indicative of deficiency. This decrease in plasma arginine was of the same order as the decrease in the dietary concentration of this amino acid, and was coupled to a 4 fold increase in the plasma glutamine concentration ($p<0.01$). High plasma glutamine concentrations must result from either an increase in synthesis, or a decrease in the utilization of this amino acid, which can occur when an amino acid becomes limiting. Because glutamine is a known scavenger of excess ammonia, it appears that ammonia detoxification had to proceed via glutamine formation in deficient animals. We have previously observed this phenomenon in piglets fed an arginine free diet (Brunton et al. 1999). In addition, as evidenced by elevated BUN levels, arginine deficient animals appear to have adapted urea cycle function to increase urea production. This may have been achieved through a reduction in arginine catabolism, as there is evidence that the catabolism of a limiting amino acid can be reduced to close to zero to compensate for a dietary deficiency (Raguso et al. 1999).

A decline in ornithine oxidation may also have helped to supply the urea cycle with additional substrate. Castillo (1994b) observed an adaptive decline in ornithine oxidation during feeding of an arginine free diet to adults. Such a decline in ornithine oxidation may explain why plasma ornithine concentrations did not fall below levels observed in sow-fed piglets (Wykes et al. 1994), although the arginine deficient diet contained only 1/3 as much arginine as is typically contained in sow's milk (Pencharz et

al. 1996). These changes in ornithine metabolism were not reflected in plasma citrulline concentrations, which were not different between diets. Again, Castillo et al's (1993a, 1994b) data is a useful comparison, because plasma citrulline values in adults were significantly higher during consumption of an arginine free diet, and appear to have been unaffected by changes in the dietary intake of arginine. A similar conundrum exists in the rat (Dhanakoti et al. 1992).

Growth rate over the short term is not a sensitive indicator of amino acid sufficiency (Wykes et al. 1994). We have previously observed that feeding diets containing a low or a high level of proline produced no differences in body weight gain over 7 days of feeding (Murphy et al. 1996). The present results reflect the insensitivity of growth parameters, as 5 days of feeding diets with an adequate or a deficient amount of arginine produced no significant differences in total, or rate of body weight gain between diet groups ($p>0.05$). However, change in body composition, with a shift between protein and fat, or an increase in body water (see section 4.4) are possible, but were not measured.

NO synthesis was apparently unaffected by the level of arginine provided in the diet. The fraction of whole body arginine flux used for NO synthesis is extremely low, and NO synthesis has been estimated to account for only 1.2% of whole body arginine flux (Castillo et al. 1996). Although NO production is critical, its synthesis may therefore have little impact on overall arginine nutrition (Reeds 2000).

We administered a large dose of isotope (100μCi/kg) in anticipation of nearly complete first pass oxidation of glutamate in the small intestine (Reeds et al. 1996). Although equal doses of proline and glutamate were infused, the SRA of glutamate was

substantially lower than the SRA of proline, because de novo synthesis is the major source of circulating glutamate. Eight hours of blood sampling proved sufficient to define the monoexponential decay curve for proline, but not for glutamate. Blood sampling was consequently extended to 24 hours, and finally to 99 hours in an attempt to define a decay curve. It became clear that there was little or no decay in the SRA of the glutamate tracer. Subsequent analysis revealed that the glutamate tracer was only 79% pure, although D-glutamate was not present. Because the majority of the tracer was present as L-glutamate, however, and there was little to no decay in the SRA of this tracer, these data still demonstrate that the decay of proline, but not of glutamate, can be modelled as a single pool system.

Isolating the radioactivity associated with glutamate proved to be extremely problematic in this analysis. The collection of radioactive derivatives was complicated by the fact that aspartate elutes 0.4 minutes prior to glutamate. Because of the proximity of these peaks, precise peak separation was difficult to achieve. The internal ^{3}H-leucine standard, used to correct for the recovery of radioactivity in samples, was also a source of uncertainty in the ^{3}H results. Upon dilution of this standard, the recovery of ^{3}H in glutamate and arginine decreased substantially. This marked reduction suggested that some of the ^{3}H from the internal standard was mixing with other radioactive fractions as they eluted from the HPLC column. When radioactive derivatives of the internal standard were collected post-column, it became apparent that this was the case. Although this mixing was small (less than 2% of the total counts found in the ^{3}H- leucine standard) and consistent, it raised uncertainty regarding the absolute number of counts of ^{3}H found in the post-column fractions. Because we observed that a consistent proportion of the

total counts in ^{3}H-leucine would have 'spilled' into the glutamate fraction, there should still have been a relative decay in the radioactivity associated with ^{3}H-glutamate. Since this decay was not present, it appears that glutamate kinetics cannot be modelled using a single pool system model.

Glutamate is extensively involved in transamination reactions and is a critical link between carbohydrate and protein metabolism (Reeds et al. 2000). Its input into the TCA cycle causes carbons from glutamate to be distributed in nearly all body cells and pools. There is recycling of label from carbon-labelled glutamate tracers via oxaloacetate and possibly via pyruvate into the mucosal α-ketoglutarate/glutamate pool (Reeds et al. 1996), a site where extensive intracellular cycling of glutamate carbon occurs (Reeds et al. 2000). Therefore, the Ra of glutamate can result from inputs into the glutamate pool from a variety of sources, and loss of glutamate is into pools from which it can return. These traits are characteristic of amino acids with multiple pools (Wolfe 1984).

Given that the metabolism of glutamate is widespread in many tissues of the body, it is not surprising that glutamate did not follow single pool kinetics. The single pool model assumes that tracer injection and sampling is from a single, homogenous, instantly mixing pool (Wolfe 1984). This assumption is not valid for glutamate because of the marked intra and extracellular compartmentation of glutamate metabolism (Reeds et al. 1996, 1997). Difficulties in trying to model a multiple pool system as a single pool arise when the interchange between pools is not rapid enough to achieve a sustained, equal concentration of tracer throughout the body (Shipley and Clarke 1972). Furthermore, when secondary pools are large, they can sequester a significant quantity of tracer. This is an obvious problem with glutamate, as its pools and actions are primarily

intracellular (Darmaun et al. 1986). The skeletal muscle glutamate pool has been estimated to be 120 times greater than the tracer-miscible glutamate pool. Based on measurements of whole blood and plasma glutamate during a 4 hour intravenous isotope infusion, Darmaun et al (1986) determined that only 4% of erythrocyte glutamate is derived from, or mixes with plasma glutamate. Consequently, an infused tracer cannot equilibrate with intracellular pools over the typically short course of most infusion experiments. Darmaun et al (1986) concluded that there is little mixing of intravenously administered glutamate tracers with intracellular pools. Therefore, the kinetics derived from the administration of glutamate tracers reflect transport rates through plasma, as opposed to whole body fluxes. Despite this information, short infusion experiments with glutamate appear to be a widely accepted means of obtaining kinetic information. It is not known whether the data generated by such short term studies describe real events.

Although the decay of glutamate did not fit the monoexponential function, the suitability of glutamate as a precursor for arginine synthesis could still be evaluated based on recovery of radioactive label from glutamate in arginine. Arginine recovery in the tritium channel did not differ from background on either diet. This finding suggests that glutamate is a very poor precursor for arginine in the neonate, a conclusion that follows from Wu et al's (1994) demonstration of low P5C synthase activity in neonatal piglet enterocytes.

Studies in adults by Berthold et al (1995) have shown that fasting effects a doubling in the contribution of arginine synthesis to plasma arginine flux, while Castillo et al (1994b) suggest that arginine homeostasis is maintained through varying the rate of arginine degradation relative to dietary intake and metabolic state. There are however,

fundamental differences between arginine metabolism in neonates and adults. Our results suggest that the partitioning of proline for arginine synthesis is regulated by the level of arginine provided in the diet, and demonstrate that proline is a very important precursor for arginine synthesis, particularly when arginine is deficient in the diet. A significantly greater proportion of arginine flux came from proline when the diet was deficient in arginine. In the instance of deficiency, more arginine must be synthesized via the P5C pathway to compensate for the lack of dietary input, while less synthesis is required when dietary arginine is sufficient. The remainder of arginine flux must be derived from the diet and endogenous protein breakdown. Although we did not measure arginine flux in this study, based on plasma amino acid concentrations, it is plausible that arginine flux is lower in the plasma of piglets fed the arginine deficient diet. Such a decrease in flux may affect our estimates of the contribution of proline to arginine synthesis on the 2 test diets. Previous work in neonates and adult males has demonstrated that arginine flux increases with the provision of greater quantities of dietary arginine (Castillo et al. 1994a,1995a, b, Beaumier et al. 1995). Murch et al (1996), however, did not find a significant difference in arginine flux between piglets intravenously fed low or higher arginine diets, although there was a trend toward higher flux with the provision of a higher level of dietary arginine. In Chapter 4.0 we present our results with respect to arginine flux in neonatal piglets fed arginine adequate or deficient diets.

In contrast to the decay of glutamate, the decay of proline fit the monoexponential function well, with r^2 values between 0.96 and 1.00. Our final objective could therefore only be achieved for proline. Although the ability to fit a line to the decay of proline is not justification for treating this system as a single pool, for practical purposes, such

simplifications can be useful and effective (Shipley and Clarke 1972). The P/F ratio of approximately 2.0 derived for proline in this study was the same for both of the test diets, however, it was twice the value obtained from a previous bolus infusion experiment conducted in our laboratory (P/F of 1.0) (Bertolo PhD Thesis 1999). This P/F value of 1.0 was subsequently tested in a prime, constant infusion experiment with good results, as it did not result in an under or an overprime of the proline pool (Bertolo et al 2002).

In this study, as well as Bertolo's (PhD Thesis 1999), the fit of the proline decay curve to the monoexponential function was excellent, with r^2 values above 0.91. The route and length of infusion, and time of plasma sampling were identical in both studies. The differences between the 2 trials included our use of [U-^{14}C]proline, versus Bertolo's [2,3-^{3}H]proline, the bolus doses administered (Wilkinson 100μCi/kg versus Bertolo 15μCi/kg), as well as the level of arginine provided in the diet (Wilkinson 0.20g/kg/d and 1.80g/kg/d versus Bertolo 1.00g/kg/d). A higher P/F ratio indicates that the proline pool is turning over more slowly, and such a result cannot be attributed to the administration of different doses of isotope. According to Said et al (1989), there is little difference in the kinetics of [5-^{3}H] and [U-^{14}C] proline, and thus we hypothesized that the difference must be due to the level of arginine provided in the various diets. We tested this hypothesis with a bolus infusion of [2,3-^{3}H]proline into 2 piglets each fed an arginine adequate or an arginine deficient diet, described in Chapter 4.0. Surprisingly, the result was identical, with P/F values of 1.79 and 2.12, respectively. The P/F ratio of 2.0 was also tested in a subsequent prime, constant infusion experiment described in Chapter 4.0. In that study, a 2:1 ratio correctly primed the proline pool during intragastric infusion of [U-^{14}C]proline in piglets fed arginine adequate and deficient diets, confirming our

hypothesis that the differences in P/F ratios were due to differences in the dietary intake of arginine, and not in the isotopes infused.

In conclusion, we have demonstrated that intragastrically fed neonatal piglets can maintain normal plasma ammonia levels on a diet containing 0.20g/kg/d of arginine. Although the decay of glutamate could not be modelled as a single pool system, the decay of proline can be modelled in this way. Our results suggest that the level of arginine provided in the diet regulates the conversion rate of proline to arginine in the neonate. Proline, but not glutamate, appears to be an important precursor for arginine synthesis in the 10 day old piglet, particularly when arginine is deficient in the diet. The kinetic data demonstrates that piglets administered arginine adequate or deficient diets require a relatively large priming dose during primed, constant infusions (P/F of 2.0), compared with animals fed an intermediate quantity of arginine. The primed, constant infusion experiment described in Chapter 4.0 was designed to build on the results from this trial, using these diets and the P/F ratios generated, to more fully examine the regulation of arginine synthesis in the neonatal intestine.

4.0 ARGININE SYNTHESIS IS REGULATED BY DIETARY ARGININE INTAKE IN THE NEONATAL PIGLET

4.1 INTRODUCTION

In the previous experiment (Chapter 3.0), I validated the arginine content of 2 experimental diets for the neonatal piglet; one designed to represent the minimum dietary inclusion level that could prevent hyperammonemia (0.20g/kg/d), and the other to provide an adequate arginine intake (1.80g/kg/d). By feeding these diets to piglets for 5 days, I observed evidence to suggest that these animals had a basal and a maximal arginine synthetic capacity. The partitioning of proline for arginine synthesis also appeared to be affected by the quantity of arginine provided in the diet, whereas glutamate seemed to be a very poor precursor for arginine synthesis in the neonatal piglet. The following study was therefore designed to elucidate whether the neonatal intestine does indeed have a basal and a maximal arginine synthetic capacity, and whether the dietary arginine intake affects the degree to which proline and glutamate are used as precursors for arginine synthesis.

Our laboratory has previously shown that arginine and proline are "co-indispensable" (Brunton et al. 1999), and that proline is a significant precursor for small intestinal arginine synthesis (Bertolo et al. 2002). Glutamate, via P5C, may also be a precursor for the synthesis of arginine in the neonate, although the data are limited. In vitro studies of arginine synthesis from glutamine in enterocytes of pre-weaning piglets suggest that this conversion is limited by low P5C synthase activity in the neonatal intestine (Wu et al. 1994). The activities of many urea cycle enzymes undergo marked changes throughout early development. P5C synthase activity, initially high in 0 and 2

day old pigs, decreases during the first week of life (Wu and Knabe 1995a). Although it remains low, P5C synthase activity increases by day 14 to 21, and again to a much greater degree in 29 to 58 day old animals (Wu 1997). However, despite the presence of enzymatic activity, no conversion of glutamine to arginine could be detected in enterocytes from piglets aged 14-21 days, while there was conversion in 0-7 day old cells (Wu et al. 1995c). Plasma cortisol concentrations may mediate many developmental fluctuations in enzyme activities, enhancing the metabolism of glutamine and arginine such that citrulline synthesis from glutamine (via induction of argininosuccinate lyase (ASL) and P5C synthase activities), and arginase activity, are increased in weaned animals (Flynn and Wu 1997).

The utilization of glutamate as a precursor for arginine synthesis in the neonate has not been thoroughly investigated. In support of measurements of low P5C synthase activity in neonatal enterocytes (Wu et al. 1994), Murphy et al (1996) did not detect any conversion of intragastrically administered glutamate to arginine in 10 day old piglets. In milk fed piglets, however, 17% of mucosal arginine flux originated from intragastrically infused glutamate (Reeds et al. 2000). Although there is little in vivo data and enzyme studies are indirect, these observations underscore the need to avoid extrapolations from older mammals to neonates, particularly in studies of arginine synthesis from glutamine. These data clearly indicate that the contribution of glutamate to arginine synthesis changes according to age. Therefore, the importance of glutamate as a precursor for arginine must be distinctly characterized at various time points during early development.

The mechanisms governing the rate of endogenous arginine synthesis in the neonate are not known. Studies in a mature rodent model suggest that renal arginine

synthesis is independent of dietary arginine intake (Dhanakoti et al. 1992). Similarily, isotopic investigations conducted in healthy men fed arginine adequate or arginine free diets suggest that de novo arginine synthesis is not increased in response to a reduced level of dietary arginine (Castillo et al. 1993a). Arginine homeostasis in the adult human therefore, appears to be maintained via altering arginine degradation relative to dietary intake and metabolic state. This hypothesis is supported by a significantly reduced rate of conversion of arginine to ornithine measured in the plasma, combined with a 46% reduction in ornithine oxidation in healthy men fed arginine free diets (Castillo et al. 1994b). Because the site of arginine synthesis differs between the adult (kidney) and the neonate (small intestine) (Wu and Knabe 1995a), the regulation of arginine synthesis should be examined separately in the neonate. Furthermore, to the authors' knowledge, the relative contributions made by proline and glutamate to arginine synthesis have never been examined under different dietary intakes of arginine in the neonate.

Although Reeds (2000) has described nitric oxide (NO) production as critical to "[maintain] the body in condition", because NO synthesis is estimated to consume only 1.2% of whole body arginine flux in adults (Castillo et al. 1996), its synthesis may have little impact on overall arginine nutrition. Furthermore, first pass splanchnic metabolism of dietary arginine in healthy adults may account for only 16% of the total daily endogenous NO production (Castillo et al. 1993c). Direct and indirect evidence, however, suggests that levels of plasma and dietary arginine can impact NO production. Indeed, low plasma arginine concentrations have been documented in infants affected with persistent pulmonary hypertension of the newborn (PPHN) and necrotizing enterocolitis (NEC) (Vosatka et al. 1994, Zamora et al. 1997, Pearson et al. 2001, Amin

et al. 2002). Because these conditions are associated with disordered NO production, it is important to determine the relationship between dietary arginine and NO production in the healthy neonate. Dietary arginine deficiency, if indeed it can impair NO production in a healthy neonate, may predispose to, or perhaps cause such conditions.

The objectives of this experiment were: 1) To quantify the maximal arginine synthetic capacity of the neonatal intestine; 2) To determine whether there is a basal level of arginine synthesis that occurs even when the dietary supply of arginine is adequate; 3) To determine whether the level of arginine provided in the diet regulates endogenous arginine synthesis and the partitioning of precursors for arginine synthesis; 4) To test if the level of dietary arginine alters whole body NO synthesis in a healthy neonate; and finally 5) To determine whether the estimate of the turnover time of the proline pool is affected by the level of dietary arginine and/or by the choice of proline tracer.

4.2 MATERIALS AND METHODS

4.2.1 Animals and Surgical Procedures

Ten male Yorkshire piglets were obtained from the University of Alberta's swine research unit. All piglets were left with the sow until 1-2 days of age, and were then transferred to the metabolic unit for immediate surgical implantation of catheters. All procedures used in this study received the approval of the University of Alberta Animal Care and Welfare Committee.

Piglets were initially anaesthetised with an intramuscular injection of acepromazine and ketamine hydrochloride, and subsequently maintained under a mixture of oxygen and halothane throughout surgery (day 0). Three silastic catheters were

installed into the stomach, femoral vein, and umbilical vein using aseptic technique (Wykes et al. 1993). A Stamm gastrostomy was performed in all piglets to install the gastric catheter, which was used for intragastric feeding and tracer infusion. The femoral vein catheter, used for blood sampling, was introduced through a common abdominal incision, and advanced into the inferior vena cava just caudal to the heart. The umbilical catheter was inserted transperitoneally into the umbilical vein, and guided to the portal-hepatic junction. Radiolabelled tracers were infused into this catheter, thereby isolating the effect of first pass gut metabolism (Bertolo et al. 1999a). Catheters were tunnelled under the skin from the point of exit on the left side of the chest, to the points of entry into the blood vessels or stomach.

Immediately following surgery, piglets were given injections of buprenorphine hydrochloride (0.05mg) (Buprenex, Reckitt and Colman Pharmaceutical Inc, Richmond, VA), trimethoprim (10mg) and sulfadoxine (50mg) (Borgal, Hoechst Roussel Vet Canada Inc, Regina, SK), and gentamicin sulfate (5mg) (Garasol, Schering-Plough Animal Health, Pointe-Claire, PQ). Gentamicin sulfate and trimethoprim and sulfadoxine injections were continued for 6 days following surgery. To ensure the patency of the umbilical catheter, a 0.9% saline solution was infused at 1mL/h throughout the study period. Animals were housed individually in circular wire-mesh cages that were cleaned daily. Cages were arranged in banks of 4, allowing visual and audio contact with other piglets. Toys were provided for environmental enrichment. The room was lit from 0800 to 2000, and the temperature was maintained at 28°C, with supplemental heat provided by heat lamps.

4.2.2 Diets

The composition of the complete and test diets are described in section 3.2.2. Immediately following surgery, the complete diet was infused intravenously via the femoral catheter at 50% of targeted intake until the morning of day 1. Diet infusion continued via the gastric catheter at 50% of full rate for 12h, 75% of full rate for 12h, and then at full rate (13.5mL/kg/h) until the morning of day 3. On the morning of day 3, piglets were randomly assigned to receive either an arginine adequate (1.80g/kg/d), or an arginine deficient (0.20g/kg/d) diet for 5 days (Figure 4.2.1).

4.2.3 Blood Sampling

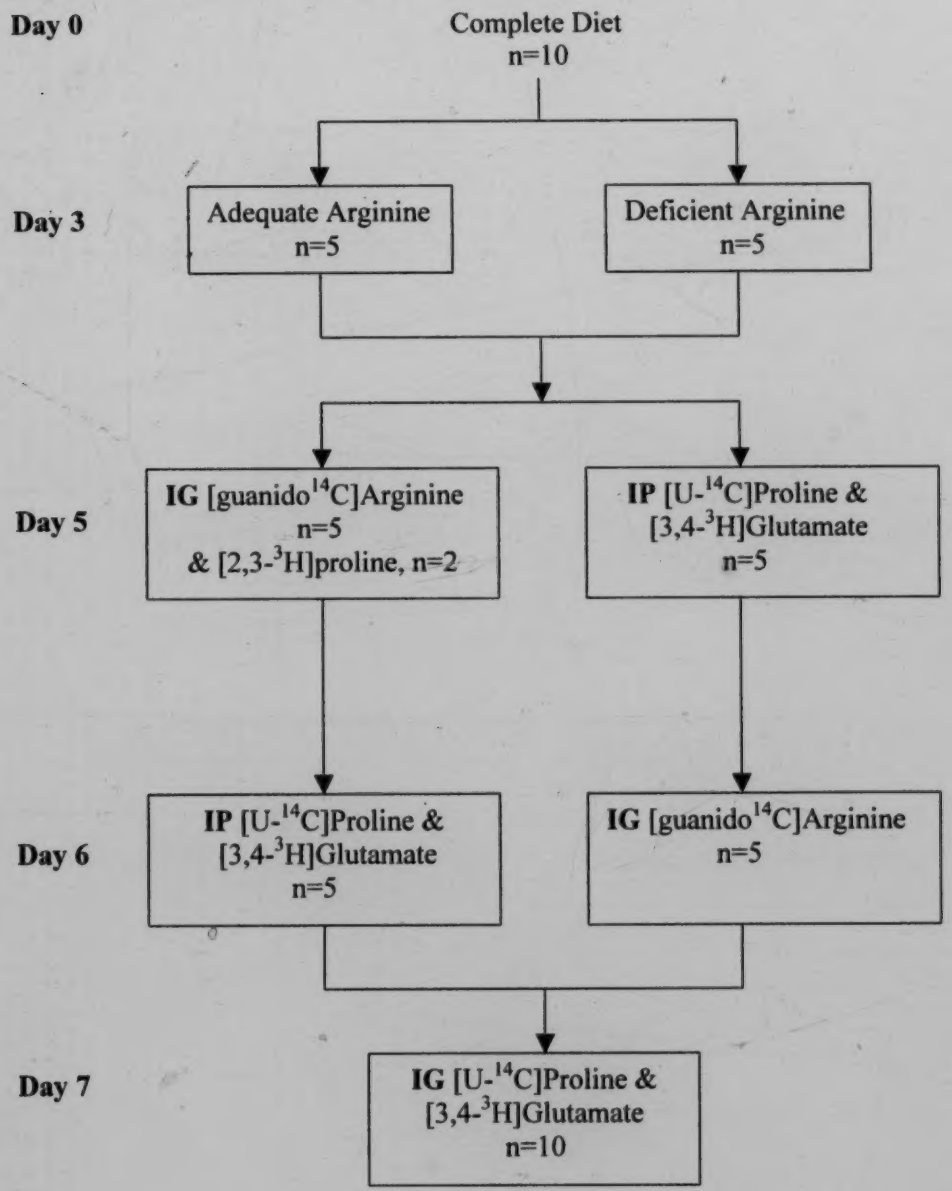
Blood samples were collected from all piglets daily for the determination of NO, blood urea nitrogen (BUN), and plasma ammonia levels. As described in section 4.2.4, additional blood samples were taken during tracer infusion on days 5, 6, and 7.

4.2.4 Constant Tracer Infusions

On the morning of day 5, arginine kinetics were determined by a primed (3μCi/kg), constant infusion (5μCi/kg/h) of a 0.9% saline solution containing 5mCi/L of L-[guanido-^{14}C]arginine via the gastric catheter. These infusion doses were chosen based on a previous experiment (Bertolo et al. 2002). Immediately prior to isotope infusion, test diets were stopped temporarily to compensate for the approximate 3mL volume of isotope to be injected. Following injection, diet was immediately restarted, and continued for the duration of blood sampling.

Tracer was infused over a 6.5 hour period, with blood sampled hourly for the first 4 hours, and every 30 minutes thereafter. In 2 of the10 animals, proline kinetics were simultaneously determined by a bolus infusion of 100 μCi/kg of a 0.9% saline solution

Figure 4.2.1 Experimental design for Chapter 4.0



containing 100 μCi/mL of L-[2,3-^{3}H]proline. An additional blood sample was taken from these animals at 8 and 12 hours.

On the morning of day 6, proline kinetics were determined by a primed (10μCi/kg), constant infusion (10μCi/kg/h) of a 0.9% saline solution containing 10mCi/L of L-[U-^{14}C]proline. Radiolabelled glutamate was simultaneously infused as a primed (5μCi/kg), constant infusion (10μCi/kg/h) of a 0.9% saline solution containing 10mCi/L of L-[3,4-^{3}H]glutamate. Tracers were infused via the umbilical catheter over a 7 hour period, with blood sampled every hour for 4 hours, and every 30 minutes thereafter. The 1mL/h infusion of 0.9% saline was discontinued during tracer infusion.

On the morning of day 7, proline kinetics were determined by a primed (20-22μCi/kg), constant infusion (10μCi/kg/h) of a 0.9% saline solution containing 10mCi/L of L-[U-^{14}C]proline. Radiolabelled glutamate was simultaneously infused as a primed (10μCi/kg), constant infusion (10μCi/kg/h) of a 0.9% saline solution containing 10mCi/L of L-[3,4-^{3}H]glutamate. Immediately prior to isotope infusion, test diets were stopped temporarily to compensate for the approximate 5mL volume of isotope to be injected. Following injection, diet was immediately restarted, and continued for the duration of blood sampling. Tracers were infused via the gastric catheter over an 8 hour period, with blood sampled every hour for 4 hours, and every 30 minutes thereafter.

In 5 animals, the order of isotope infusion on days 5 and 6 was reversed. These 5 animals were given a primed, constant infusion of intraportally administered L-[U-^{14}C]proline and L-[3,4-^{3}H]glutamate on day 5, and a primed constant infusion of intragastrically delivered L-[guanido-^{14}C]arginine on day 6 of the protocol. This reversal

allowed additional time for the washout of intraportally infused proline and glutamate tracers prior to intragastric infusion on day 7 of the same isotopes.

4.2.5 Analytical Procedures

Plasma amino acids and the specific radioactivity (SRA) of plasma glutamate, proline, arginine, hydroxyproline, glutamine, citrulline, ornithine, and urea were measured by reverse-phase HPLC using phenylisothiocyanate derivatives as previously described (House et al. 1997). Two internal standards, norleucine and L-[4,5-^{3}H]leucine (Amersham Pharmacia Biotech, St. Louis, MO), were added to each 300μl plasma sample. Post-column radioactive derivatives were collected in 2 mL fractions to which 14 mL of scintillant was added. Radioactive fractions were counted on a liquid scintillation counter using the dual isotope counting method for ^{3}H and ^{14}C. On days 6 and 7 of the infusions, SRA values at plateau were corrected for background SRA values measured on the morning of days 6 and 7 respectively.

Plasma ammonia concentrations were determined every 24 hours from the start of test diet infusions. The colorimetric assay used in the analysis was based on the amination of 2-oxoglutarate to glutamate with simultaneous oxidation of NADPH (Sigma Procedure No. 171-UV, Sigma Diagnostics, St. Louis, MO). Plasma urea concentrations were measured daily and during tracer infusions, using a spectrophotometric assay kit in which ammonia was liberated from urea by enzymatic hydrolysis (Sigma Chemical, St. Louis, MO).

Plasma NO concentration was determined via chemical reduction of nitrate to nitrite, followed by spectrophotometric analysis of total nitrite using Griess reagent (Oxis International Inc, Portland, OR).

4.2.6 Calculations

Amino acid concentrations were calculated as:

[Amino acid (μmol/L)] = [(Amino acid peak area ÷ Norleucine peak area) * 0.10μmol norleucine added] ÷ volume of plasma in L

Plasma SRA's were calculated as follows:

SRA (dpm/μmol) = Amino acid radioactivity (dpm/200μL) ÷ [Amino acid (μmol/200μL)]

Plateau in plasma SRA was calculated as the mean of the SRA for the time points in the plateau period. All plateaux included at least 4 time points except for the conversion of arginine to urea, which included 3. SRA ratios at plateau of product to precursor were used to determine the fractional net conversion of precursor to product.

Fractional net conversion (%) = ($SRA_{\text{amino acid product}}$ ÷ $SRA_{\text{infused amino acid}}$) x 100

Fluxes of infused amino acids (arginine, proline, and glutamate) were calculated as:

Flux (μmol/kg/h) = Constant infusion rate (dpm/kg/h) ÷ $SRA_{\text{infused amino acid}}$ at plateau

Values for flux and fractional net conversion were used in the calculation of molar rates of conversion of precursor (proline, glutamate) to arginine, following the approach of Beaumier et al. (1995).

$Q_{\text{precursor to product}}$ (μmol/kg/h) = Fractional net conversion (%) x $\text{Flux}_{\text{IG arg}}$ (μmol/kg/h)

Small intestinal (gut) first pass contribution to $Q_{\text{precursor to product}}$ was calculated as the difference between $Q_{\text{precursor to product}}$ during IG and IP infusions.

Calculations for bolus infusions were carried out as described in section 3.2.6 using the single-pool method detailed by Wolfe (1992).

4.2.7 Statistical Analyses

Statistical analyses were performed using the mixed model of SAS Version 8.3 (SAS Institute, Cary, NC). The dependent variables NO, ammonia, and BUN were analysed using a repeated measures analysis. The fixed effects were treatment (arginine adequate or deficient diet), while days and pig were the random effects. In all statistical models the kenward-roger option was used to estimate denominator degrees of freedom. The variance-covariance matrix chosen for each analysis was the best fit model based on the Schwarz's Bayesian Criterion. Least squares means were separated using the pdiff option when fixed effects were significant ($p<0.05$). A one-tailed test was used to compare plasma ammonia and BUN values between diet groups, because we expected these values to be significantly higher in arginine deficient compared to arginine adequate animals. Given the lack of comparable studies, a prediction could not be made regarding the result for the NO analysis, and hence a two-tailed test was employed. A two-way analysis of variance with interaction was used to test whether the SRA values calculated during the tracer infusions on day 7 were significantly affected by the order of isotope infusion on days 5 and 6 (SAS Version 8.3, SAS Institute Inc, Cary, N.C.).

All other comparisons between diet groups were performed using a two-tailed t-test (SAS Version 8.3, SAS Institute Inc, Cary, N.C.). Paired t-tests were used for tests of significance within diet groups. Data were considered significantly different if $p<0.05$.

4.3 RESULTS

The health status of the animals was not visibly impacted by the feeding of a diet with a deficient or an adequate quantity of arginine. There was no significant difference between the two diet groups in body weight upon arrival or at the time of initiation of the test diets ($p>0.05$) (Table 4.3.1). Total and rate of weight gain were not different during feeding of complete diet from day 0-3 ($p>0.05$), however total ($p=0.03$) and rate ($p=0.01$) of weight gain were significantly higher from day 3-7 in pigs receiving the arginine deficient diet.

The initiation of the arginine deficient diet precipitated a rise in plasma ammonia levels in the animals receiving this diet. The morning after test diet infusions were initiated, 2 animals had plasma ammonia concentrations that exceeded 270μmol/L, a level our laboratory has previously deemed to be a critical value for plasma ammonia levels (Brunton et al. 1999). On day 7 of the study, plasma ammonia concentrations in a 3rd animal exceeded this critical value. These 3 animals did not, however, exhibit any external symptoms of ammonia toxicity. During test diet infusion, plasma ammonia levels were significantly greater in piglets receiving the diet containing a deficient level of arginine than in animals given an adequate quantity of arginine ($p<0.05$, one-tailed test) (Figure 4.3.1). BUN levels were significantly greater in piglets fed arginine deficient diets ($p<0.04$, one-tailed test) (Figure 4.3.2).

Piglets administered a diet with a deficient level of arginine had significantly lower plasma arginine concentrations than piglets provided with an adequate level of arginine ($p<0.01$) (Table 4.3.2). Plasma ornithine ($p<0.01$) and hydroxyproline ($p<0.05$) were also significantly lower in piglets fed the deficient diet. Conversely, plasma

Table 4.3.1 Growth performance of neonatal piglets intragastrically fed adequate (1.80g/kg/d) or deficient (0.20g/kg/d) quantities of arginine for 5 days

	Adequate Arg	Deficient Arg	SEM
Weight (study d0) (kg)	1.62	1.66	0.05
Weight Gain Complete Diet (d0-3) (kg)	0.31	0.29	0.03
Rate of Weight Gain Complete Diet (d0-3) (g/kg/d)	93	85	10
Weight at Initiation of Test Diets (d3) (kg)	1.93	1.95	0.07
Weight Gain Test Diet (d3-7) (kg)	0.72	0.89[a]	0.05
Rate of Weight Gain Test Diet (d3-7) (g/kg/d)	82	99[a]	4

n=10

[a]$p<0.05$ compared to piglets fed adequate arginine diets

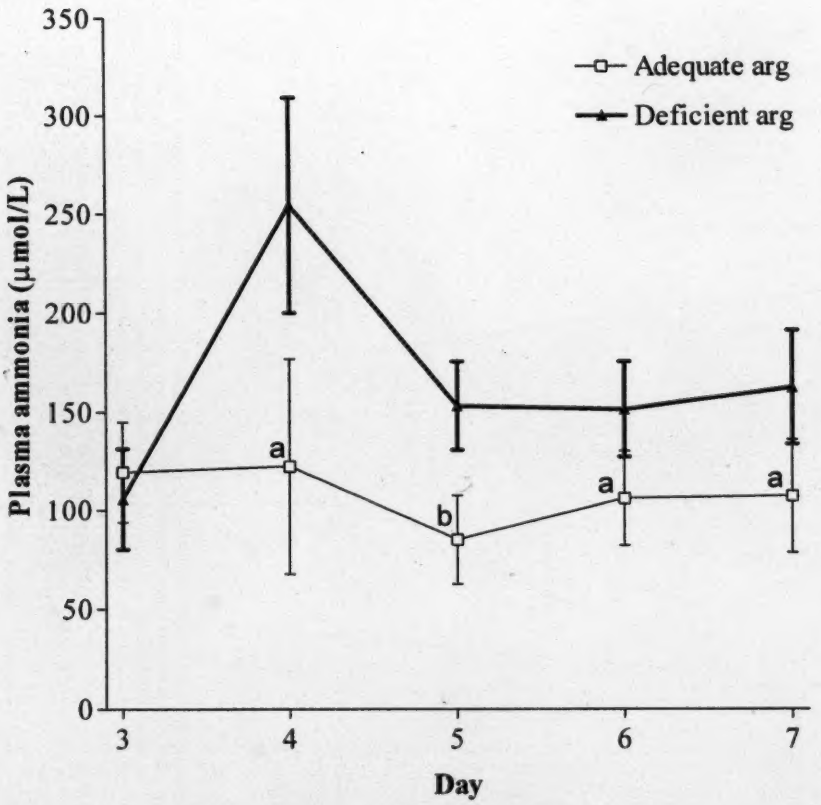


Figure 4.3.1 Mean plasma ammonia concentrations (µmol/L) of piglets fed an arginine adequate (1.80g/kg/d) or deficient (0.20g/kg/d) diet for 5 days. Plasma ammonia concentrations of piglets fed an arginine deficient diet were significantly higher than those of piglets fed an adequate diet ($p<0.05$, one-tailed test) as determined by a repeated measures analysis using the mixed model of SAS Version 8.3 (SAS Institute, Cary, NC). Data are means ± SEM, n=10. Least squares means were separated using the pdiff option when fixed effects were significant, superscripts are used to indicate the result of these comparisons.
[a]Point is different from arginine deficient on day 4.
[b]Point is different from arginine deficient on day 4, 5, 6, 7.

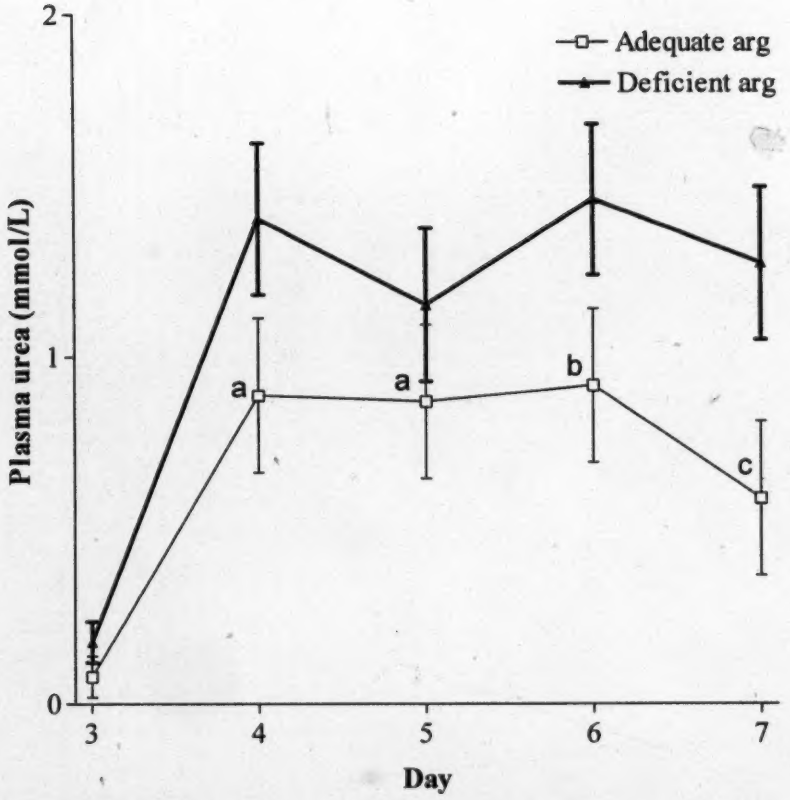


Figure 4.3.2 Mean plasma urea concentrations (mmol/L) of piglets fed an arginine adequate (1.80g/kg/d) or deficient (0.20g/kg/d) diet for 5 days. Plasma urea concentrations of piglets fed an arginine deficient diet were significantly higher than those of piglets fed an adequate diet ($p<0.04$, one-tailed test) as determined by a repeated measures analysis using the mixed model of SAS Version 8.3 (SAS Institute, Cary, NC). Data are means ± SEM, n=10.
Least squares means were separated using the pdiff option when fixed effects were significant, superscripts are used to indicate the result of these comparisons.
[a]Point is different from arginine deficient on day 4, 6.
[b]Point is different from arginine deficient on day 6.
[c]Point is different from arginine deficient on day 4, 5, 6, 7.

Table 4.3.2 Plasma amino acid concentrations (μmol/L) of neonatal piglets intragastrically fed adequate (1.80g/kg/d) or deficient (0.20g/kg/d) quantities of arginine for 5 days

Amino Acid	Adequate Arg	Deficient Arg	SEM	Sow-fed Reference Range[1]
		μmol/L		
Indispensable				
Histidine	83	87	11	47-121
Isoleucine	106	116	10	81-143
Leucine	**299**	**264**	24	102-175
Lysine	**649**	**684**	41	77-317
Methionine	**129**	52[a]	16	33-74
Phenylalanine	**119**	**105**	9	34-86
Threonine	**1445**	**536**[a]	113	214-500
Tryptophan	58	57	4	
Valine	271	254	22	175-318
Conditionally Indispensable				
Arginine	**396**	**33**[a]	18	50-267
Cysteine	15	11	4	
Glutamine	79	339[a]	32	
Glycine	978	**2801**[a]	120	450-976
Proline	890	729	110	304-890
Taurine	144	148	16	
Tyrosine	99	88	11	88-231
Other				
Alanine	708	901	96	413-1015
Aspartate	15	35	13	22-63
Citrulline	132	111	14	73-151
Glutamate	137	157	19	92-217
Hydroxyproline	130	96[a]	11	
Ornithine	**397**	87[a]	45	79-195
Serine	235	**1933**[a]	159	137-442

n=10

[1]Data from Wykes et al (1994)

[a]$p<0.05$ compared to piglets fed adequate arginine diets

Bolded numbers indicate these values were outside the sow-fed reference range

glutamine concentrations were significantly higher in piglets receiving the arginine deficient diet ($p<0.01$).

Plasma concentrations of serine and glycine were significantly greater in piglets given the arginine deficient diet ($p<0.01$), while methionine and threonine ($p<0.01$) were significantly lower in piglets fed a deficient level of arginine (Table 4.3.2).

The fluxes for arginine and proline are presented in Table 4.3.3. Proline flux was not significantly affected by the quantity of arginine provided in the diet, regardless of the route of infusion (IP vs IG) ($p>0.05$). Arginine flux, determined from intragastric tracer infusion, was significantly lower in animals receiving a deficient quantity of arginine ($p<0.01$). The values for glutamate flux were not affected by diet or route of infusion ($p>0.05$) (Table 4.3.3).

The net flux from proline to arginine ($Q_{pro \rightarrow arg}$) is presented in Table 4.3.4 for IP and IG infusions. The difference between the net flux calculated by these routes of infusion represents the first pass small intestinal contribution to net flux. IP and IG net $Q_{pro \rightarrow arg}$ were significantly greater in piglets receiving an inadequate supply of arginine from dietary sources ($p<0.01$), although diet did not significantly affect first pass intestinal contribution to $Q_{pro \rightarrow arg}$ ($p>0.05$). Despite a greater level of synthesis in deficient animals, the total available arginine (intake, 0.20g/kg/d + synthesis, 0.68g/kg/d) in deficient animals was significantly lower than the estimated arginine requirement of 1.80g/kg/d ($p<0.01$). The first pass intestinal net contribution to $Q_{pro \rightarrow arg}$ in animals fed an adequate level of arginine was significantly greater than $Q_{pro \rightarrow arg}$ during IP tracer infusion ($p<0.01$). In piglets fed a deficient level of arginine, there was no significant

Table 4.3.3 Plasma proline, glutamate, and arginine fluxes (μmol/kg/h) of neonatal piglets intragastrically fed adequate (1.80g/kg/d) or deficient (0.20g/kg/d) quantities of arginine for 5 days

	Adequate Arg	Deficient Arg	SEM
IP Pro Flux	441	388	22
IG Pro Flux	431	394	24
IP Glu Flux	298	516	147
IG Glu Flux	447	549	215
IG Arg Flux	1011	590[a]	62

n=10

[a]$p<0.01$ compared to piglets fed adequate arginine diets

Table 4.3.4 Plasma net $Q_{pro \rightarrow arg}$ (μmol/kg/h) of neonatal piglets intragastrically fed adequate (1.80g/kg/d) or deficient (0.20g/kg/d) quantities of arginine for 5 days

$Q_{pro \rightarrow arg}$	Adequate Arg	Deficient Arg	SEM
IP μmol/kg/h (g/kg/d)	31 (0.13)	95 (0.40)[a]	6 (0.02)
% of Requirement[1]	7	22[a]	1
% of Arg Flux	3	16[a]	1
IG μmol/kg/h (g/kg/d)	85 (0.36)[b]	162 (0.68)[ab]	13 (0.05)
% of Requirement[1]	20[b]	38[ab]	3
% of Arg Flux	8[b]	27[ab]	1
Intestinal Contribution to Flux μmol/kg/h (g/kg/d)	54 (0.23)[b]	68 (0.28)	13 (0.05)
% of Requirement[1]	13[b]	16	3
% of Arg Flux	5[b]	11[a]	2

n=10

[1]Requirement was defined as 1.80g/kg/d (10333 μmol/kg/d) of arginine (Brunton et al. 2001, Wilkinson et al. 2002)

[a]$p<0.05$ compared to piglets fed adequate arginine diets

[b]$p<0.01$ compared to IP $Q_{pro \rightarrow arg}$

difference between the contribution made by first pass gut metabolism to $Q_{pro \rightarrow arg}$ compared to $Q_{pro \rightarrow arg}$ measured during IP tracer infusion ($p>0.05$). Table 4.3.4 also presents net $Q_{pro \rightarrow arg}$ as a percentage of the estimated arginine requirement (1.80g/kg/d) and as a percentage of arginine flux (fractional net conversion).

The recovery of radioactive label from glutamate in arginine was very small, and was negligible (<20 decays per minute (dpm)) when corrected for background dpm measured in the animals (residual from the previous days' isotope infusion), the scintillation counter, and in the internal ^3H leucine standard. The net flux from glutamate to arginine ($Q_{glu \rightarrow arg}$) was therefore deemed to be biologically insignificant.

Fractional net conversions of proline to hydroxyproline, citrulline, glutamine, and ornithine are presented in Table 4.3.5. When arginine was provided at an adequate level in the diet, the fractional net conversion of proline to citrulline ($p=0.05$) and ornithine ($p<0.01$) was significantly greater on first pass by the gut (= IG - IP conversion) than during IP infusions. These parameters were not different from each other in arginine deficient animals ($p>0.10$). With the provision of an adequate quantity of dietary arginine, the first pass gut fractional net conversion of proline to hydroxyproline was significantly lower than the net conversion measured during IP infusion ($p=0.01$), a trend that was also exhibited in deficient pigs ($p=0.09$). The first pass intestinal net conversion of proline to glutamine was not different from the net conversion observed during IP infusion in adequate pigs ($p>0.05$). In deficient pigs, first pass gut conversion of proline to glutamine was smaller than conversion during IP infusion ($p<0.01$).

Although the flux of the metabolic intermediates was not quantified, an examination of the fractional net conversions of proline to hydroxyproline, citrulline,

Table 4.3.5 Plasma fractional net conversion (%) of proline to hydroxyproline, citrulline, glutamine, and ornithine in neonatal piglets intragastrically fed adequate (1.80g/kg/d) or deficient (0.20g/kg/d) quantities of arginine for 5 days, determined during L-[U-^{14}C]proline infusions

Fractional Net Conversion of Pro to... (%)	Adequate Arg	Deficient Arg	SEM
IP			
Hydroxyproline	11	13	2
Glutamine	5	2	1
Citrulline	22	26	3
Ornithine	5	16[a]	2
IG			
Hydroxyproline	12	15[b]	1
Glutamine	11[d]	3[a]	1
Citrulline	62[c]	61[c]	4
Ornithine	20[c]	37[ac]	1
Intestinal Contribution			
Hydroxyproline	1[c]	2[d]	2
Glutamine	6	0.3[bc]	2
Citrulline	40[c]	34	5
Ornithine	15[c]	22[a]	2

n=10
[a]$p<0.05$ compared to piglets fed adequate arginine diets
[b]$p<0.10$ compared to piglets fed adequate arginine diets
[c]$p<0.05$ compared to IP fractional net conversion
[d]$p<0.10$ compared to IP fractional net conversion

Table 4.3.6 Plasma fractional net conversion (%) of arginine to urea in neonatal piglets intragastrically fed adequate (1.80g/kg/d) or deficient (0.20g/kg/d) quantities of arginine for 5 days, determined during L-[U-^{14}C]proline infusions

	Adequate Arg	Deficient Arg	SEM
IG Fractional Net Conversion of Arg to Urea (%)	68	14[a]	3

n=10
[a]$p<0.01$ compared to piglets fed adequate arginine diets

glutamine, and ornithine is nevertheless instructive (Table 4.3.5). The fractional net conversion of proline to ornithine was lower in arginine deficient pigs during IP and IG infusions ($p<0.01$) and on first pass by the gut ($p=0.05$) compared to arginine adequate pigs. Net conversion of proline to glutamine was only significantly different between diets when gut first pass metabolism was included (IG $p<0.01$ and first pass intestinal contribution $p=0.06$). No significant differences were detected in the fractional net conversion of proline to hydroxyproline or citrulline ($p>0.05$), although there was a trend ($p<0.10$) for the whole body (IG) net conversion of proline to hydroxyproline to be greater in piglets fed a deficient diet. The fractional net conversion of arginine to urea was significantly greater in piglets receiving an adequate dietary supply of arginine during IG arginine infusion ($p<0.01$) (Table 4.3.6).

Fractional net conversions of glutamate to hydroxyproline, glutamine, citrulline, and ornithine were not calculated. These data were considered unreliable because of our inability to precisely isolate the glutamate peak, and because of the high error associated with glutamate flux values.

The plateaux in plasma SRA achieved on day 7 of the protocol were not significantly impacted by the order of isotope infusion on days 5 and 6 ($p>0.05$).

There was a trend ($p<0.09$) for plasma NO concentrations to be affected by the level of arginine provided in the diet (Figure 4.3.3).

For the arginine adequate diet, a P/F ratio of 1.79 was calculated for proline. This value was similar to the ratio determined in deficient piglets of 2.12.

4.4 DISCUSSION

In this study, there was a significant difference in the rate of body weight gain

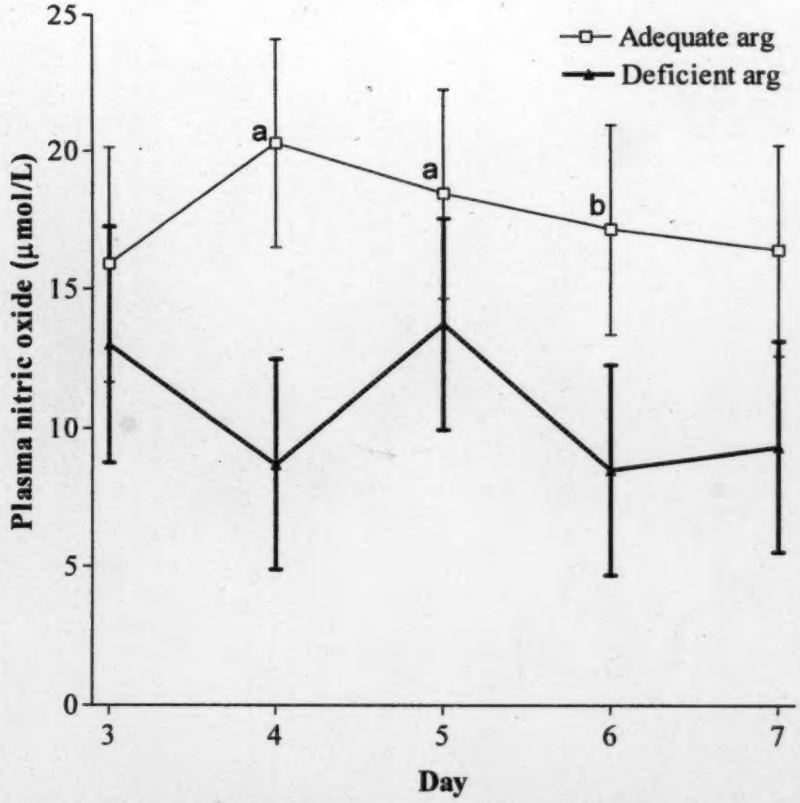


Figure 4.3.3 Mean plasma nitric oxide concentrations (µmol/L) of piglets fed an arginine adequate (1.80g/kg/d) or deficient (0.20g/kg/d) diet for 5 days. There was a trend ($p<0.09$) for plasma nitric oxide concentrations to be affected by the level of arginine provided in the diet, as determined by repeated measures analysis using the mixed model of SAS Version 8.3 (SAS Institute, Cary, NC). Data are means ± SEM, n=10.

[a]Point is different from arginine deficient on day 4, 6, 7.

[b]Point is different from arginine deficient on day 6.

between animals intragastrically infused with diets containing adequate or deficient levels of arginine. This difference was not, however, in the expected direction, as the piglets fed a deficient diet gained significantly more body weight than those fed an adequate arginine diet. The data of House et al (1994) is instructive in this respect. In that study, glutamine supplementation to TPN tended to increase live body weight gain in neonatal piglets via an increase in total body water, and in particular extracellular fluid (ECF) expansion. The formation of glutamine from glutamate and ammonia is an important physiologic means of scavenging excess ammonia. Because the urea cycle function of the deficient arginine diet group was challenged by an inadequate arginine intake, the animals exhibited plasma glutamine concentrations that were 4 fold greater than those of piglets fed an adequate level of the amino acid. Therefore, high plasma glutamine concentrations in deficient animals may have induced an expansion in the ECF, resulting in greater body weight gain.

In contrast to the above results, when diets containing an adequate or a deficient level of arginine were fed to 6 animals in the bolus infusion trial described in Chapter 3.0, the rate of weight gain over the course of test diet infusion was not significantly different between diet groups. It is difficult to explain why such a difference, if indeed it is real, could exist between two similar protocols. Because the statistical power of a comparison of 10 piglets is greater than one of 6 piglets, this discord may be statistical in nature. There was also an apparent difference between plasma glutamine and BUN levels between the two studies. In arginine deficient animals, plasma glutamine concentrations appear to be higher, and plasma urea lower, in this study than in the first one, although plasma ammonia concentrations are comparable. The higher glutamine concentrations in

this trial may account for the difference in rates of body weight gain, by promoting additional expansion of the ECF (House et al. 1994). Given the relatively small n from the first study, and the absence of body composition data from either study, the specific reason for the discrepancy in rates of body weight gain during test diet infusions cannot be determined.

Plasma arginine concentrations in sow-fed piglets reflect the low arginine intake from sow's milk relative to requirements for protein deposition (Pencharz et al. 1996). Piglets receiving the adequate arginine diet exhibited plasma arginine concentrations that were 40% greater than the highest values observed in sow-fed animals (Wykes et al. 1994). On the other hand, animals fed a deficient diet had plasma arginine concentrations that were 90% lower than those observed in the adequately fed animals, mimicking the decline in the dietary arginine concentration. Because BUN was elevated in this diet group, it is likely that arginine oxidation was reduced to provide additional substrate for urea synthesis. Despite plasma arginine concentrations that were much lower than the sow-fed reference, plasma ornithine concentrations of the arginine deficient piglets did not drop below levels characteristic of sow-fed piglets, although they were significantly lower than those of adequately fed animals. These observations are similar to those made by Castillo et al. (1994b), who saw a decline in ornithine oxidation in healthy men fed arginine free diets. A reduction in ornithine oxidation could help to preserve urea cycle substrate in the absence of sufficient dietary arginine. Plasma citrulline concentrations were unaffected by dietary arginine deficiency, a phenomenon that has also been documented in rats (Hartman and Prior 1992), 20-50kg pigs (Prior and Gross 1995), and in adult humans (Castillo et al. 1995b).

As anticipated, plasma glutamine concentrations were a reliable indicator of arginine deficiency. Because the availability of arginine was limiting for urea cycle function in arginine deficient animals, ammonia detoxification had to proceed via glutamine formation, thereby elevating plasma concentrations. Piglets fed a deficient level of arginine had significantly lower plasma hydroxyproline concentrations than piglets fed an adequate quantity of arginine. We have previously observed a progressive decline in the plasma hydroxyproline concentrations of enterally fed piglets when the dietary intake of tryptophan was gradually decreased (Cvitkovic MSc Thesis 2000). Hydroxyproline is formed from the hydroxylation of proline after it is incorporated into protein, and thus hydroxyproline in its free form can only arise from protein degradation (Wolfe 1992). Because arginine was limiting for protein synthesis in the animals fed the deficient diet, less proline may have been incorporated into protein in these piglets, assuming no change in whole body protein breakdown. A reduction in protein synthesis would decrease the hydroxylation of proline to hydroxyproline, and therefore plasma free hydroxyproline concentrations would also decrease. On the other hand, high alanine concentrations in deficient pigs ($p=0.19$), a known scavenger of excess ammonia, may have stimulated hydroxyproline consumption through the serine hydroxymethyltransferase pathway, generating additional serine and glycine (Lowry et al. 1985). The former explanation is however, the most plausible.

Piglets fed a deficient level of arginine had significantly greater plasma glycine levels than adequately fed piglets. Additional serine was included in the arginine deficient diets to make the diets isonitrogenous, and this serine may have stimulated glycine production via serine hydroxymethyltransferase. Alternatively, there may have

been a reduction in the utilization of glycine during collagen formation in arginine deficient animals.

Glycine can stimulate threonine oxidation via the glycine dependent pathways of threonine dehydrogenase and threonine aldolase (House et al. 2001). Therefore, the very high concentrations of glycine and serine may have promoted threonine oxidation in deficient animals, resulting in the lower plasma threonine concentrations. Because our diet contains an excess of threonine relative to requirement (Bertolo et al. 1998) however, the plasma threonine concentration of piglets fed the deficient arginine diet was still higher than the sow-fed reference mean, and was unlikely to be a limiting factor in this diet. In addition, there may have been an expansion in the ECF of deficient piglets and consequently these animals may have had a larger plasma fluid compartment. Lower plasma concentrations of some amino acids may therefore have arisen from this hypothesized dilution of plasma amino acids.

Finally, plasma methionine concentrations were significantly lower in piglets fed the arginine deficient diet, although cysteine and cystathione concentrations were unaffected. The reason for this increase is not known, however it may reflect an increase in remethylation from homocysteine. There is a recognized relationship between this pathway and arginine metabolism, because high homocysteine decreases NO synthesis (Zhang 2000). Furthermore, methionine plays an important role in arginine metabolism via its donation of methyl groups during creatine and polyamine synthesis from arginine. The quantitative significance of this relationship should not be underestimated. Creatine synthesis has been estimated to consume approximately 70% of all physiologically labile methyl groups, more than all other methylation reactions combined (Wyss and

Wallimann 1994). This pathway may also contribute to the ability of arginine plus glycine to alleviate the toxic effects of excess methionine in rats (Brown and Allison 1948), while worsening the growth depression of chicks fed diets limiting in methionine (Keshavarz and Fuller 1971). These findings suggest that there may be a previously underappreciated relationship between these two amino acids.

The first major objective of this work was to quantify the maximum arginine synthetic capacity of the neonatal intestine. In order to quantify maximum synthesis, we had to provide the maximum stimulus to synthesis, namely, a deficient level of dietary arginine that would still enable long term survival of the animal. We have previously established that 0.20g/kg/d of arginine is the minimal dietary arginine inclusion level that can prevent hyperammonemia (Chapter 3.0). Although piglets fed a deficient level of arginine had higher plasma ammonia concentrations than animals fed an adequate level of the amino acid, they sustained sufficient urea cycle function to maintain moderate ammonia levels. Because arginine was limiting for protein synthesis, arginine deficient piglets had to dispose of more ammonia than arginine adequate animals. This was accomplished by increased generation of urea and glutamine. The rate of ammonia disposal was not commensurate with its rate of production, however, as evidenced by the instances of hyperammonemia. These data confirm that 0.20g/kg/d of arginine is indeed a deficient arginine intake for the neonatal piglet. Furthermore, because past intragastric administration of 0.15g/kg/d of arginine led to severe hyperammonemia within 8 hours of feeding (Brunton et al. unpublished), 0.20g/kg/d appears to be acceptable as the minimal dietary inclusion level for arginine.

Despite 2 fold greater whole body arginine synthesis in deficient compared to adequately fed animals (Table 4.3.4), the total available arginine (intake, 0.20g/kg/d + synthesis, 0.68g/kg/d) in the deficient animals was only half of the estimated arginine requirement of 1.80g/kg/d. Therefore, intestinal arginine synthesis was not sufficiently upregulated to compensate for the dietary arginine deficiency. Indeed, it is interesting that we measured metabolic indications of arginine deficiency in pigs with 0.88g/kg/d of available arginine, given that the estimated NRC requirement is 0.38g/kg/d (1998). We believe that the administration of the deficient arginine diet provided a near maximal challenge to the arginine synthetic capacity of these piglets, and therefore we have observed the maximum arginine synthetic capacity of the neonatal piglet intestine in this model. Because plasma proline concentrations did not decline in these piglets, arginine synthesis was not limited by supply of dietary proline, but by insufficient de novo synthetic capabilities. In conditions that increase the metabolic demand for arginine beyond this maximum arginine synthetic capacity, dietary arginine supplementation is necessary.

Our second objective was to quantify whether there is a basal level of arginine synthesis that occurs even when there is an adequate supply of arginine. This objective was achieved by providing arginine in the adequate diet at our hypothesized whole body arginine requirement of 1.80g/kg/d (Brunton et al. 2001, Wilkinson et al. 2002). Theoretically, arginine synthesis should cease under such conditions. These piglets, however, synthesized 0.36g/kg/d of arginine, about half as much as deficient pigs.

It is possible that the quantity of arginine provided in the adequate diet was not sufficient to satisfy the arginine requirement of these piglets. In this instance, the

arginine synthesis observed would not represent a basal level of synthesis, but would be a physiologic means of satisfying the true arginine requirement. As described in Chapter 3.0, recent results from our laboratory (Brunton et al. 2001, Wilkinson et al. 2002, Bertolo et al. 2002) suggested to us that the total metabolic demand for arginine was satisfied around 1.80g/kg/d, a level already 4.5 times greater than the current NRC estimated dietary requirement of 0.38g/kg/d (1998), and over 2.5 times the arginine intake from sow's milk (0.66g/kg/d) (Pencharz et al. 1996). Although the whole body arginine requirement for the neonate has not been established, there is no evidence to suggest that 1.80g/kg/d is an inadequate intake for TPN fed piglets (Wilkinson et al. 2002). TPN fed animals are an ideal model for elucidating the whole body arginine requirement because a lack of first pass intraluminal arginine precursors, and gut atrophy preclude arginine synthesis. On the basis of these previous observations, we are reasonably assured that 1.80g/kg/d of arginine is a metabolically sufficient intake for the neonatal piglet. We have therefore demonstrated that there is a basal and obligatory rate of arginine synthesis in the neonatal piglet.

The dietary regulation of, and the partitioning of precursors for arginine synthesis formed the third objective in this study. Net proline flux was not different between the two diets (Table 4.3.3), and therefore it did not affect estimates of arginine synthesis from proline. Proline flux was also not affected by the route of isotope infusion (IP vs IG), although we have previously observed a small, but significant difference in proline flux using these routes of infusion (Bertolo et al. 2002). Arginine flux, as anticipated, was very different between the two diets, however this difference is accounted for in the calculation of $Q_{pro \rightarrow arg}$ (section 4.2.6). Castillo et al (1994a) have also verified that the

fed state plasma arginine flux in adults is partially dependent upon the contribution of dietary substrate.

Although renal arginine synthesis does not appear to be under dietary regulation in the adult human (Castillo et al. 1993a, 1994a) or in the rat (Dhanakoti et al. 1992), our results suggest that whole body $Q_{pro \rightarrow arg}$ in the neonate is under dietary regulation. When challenged by an inadequate supply of dietary arginine, piglets were able to upregulate net synthesis of arginine from proline. On a whole body level, the net quantity of arginine synthesized from proline doubled in deficient compared to adequately fed piglets, and tripled during IP infusions (Table 4.3.4). The quantity of arginine synthesized by the gut on first pass was, however, not different between diet levels. This suggests that first pass intestinal arginine synthesis occurred at its maximum capacity even when the dietary supply of arginine was adequate. Because first pass gut conversion of proline to arginine was already operating at its maximum capacity, piglets provided with a deficient quantity of arginine in the diet upregulated conversion of proline to arginine by some other mechanism, to match the level of conversion occurring on first pass by the gut.

The intraportal infusion of tracers was developed in our laboratory to isolate first pass gut metabolism (Bertolo et al. 1999a). We have previously used this method to demonstrate that first pass gut conversion of proline to arginine is more important than first pass hepatic conversion in the neonate (Bertolo et al. 2002), however we have not investigated the contribution of the liver or of other tissues to arginine synthesis. Increased conversion of proline to arginine during IP infusions may result from increased arterial extraction of proline for arginine synthesis by the healthy gut. This arterial

extraction of proline may explain why, in an earlier study, piglets intragastrically fed an arginine and proline free diet exhibited a less severe rise in plasma ammonia than piglets fed the same diet intravenously (Brunton et al. 1999). Because our data suggests that dietary glutamate is not an important precursor for arginine in piglets of this age, it is possible that the healthy gut of the intragastrically fed animals was extracting arterial proline for conversion to arginine.

IP tracer infusion only excludes first pass amino acid metabolism by the gut, and therefore we cannot ignore the possibility that tissues other than the gut, including the liver and the kidney, may have played a role in the upregulation of net $Q_{pro \rightarrow arg}$ detected during IP infusions. In the adult, the complete urea cycle is expressed only in the liver (Morris 1992), although in the neonate it is also expressed in the intestine. There is ready cycling between proline and arginine in both the liver and the intestine (Jones 1985, Brunton et al. 1999), however the liver is considered to be the site of urea production, due to the low activity of intestinal arginase (Wu et al. 1995b). Indeed, the liver contains a distinct isozyme of the arginase enzyme, considered to be primarily catabolic and ureagenic (Iyer et al. 1998). The activity of the arginase enzyme plays a key role in regulating arginine production. Hepatic arginase activity is very high, much higher than it is in the intestine and kidney, and therefore there is almost no hepatic arginine release in adults (Beaumier et al. 1996), young pigs (Edmonds et al. 1987b) and neonatal and mature rats (Herzfeld and Raper 1976, Windmueller and Spaeth 1981). This must also be true of preterm neonates, because the activity of hepatic arginase is similar between preterm infants and adults, although it declines during infancy and childhood (Oyanagi et al. 1980).

It is clear that the liver does not provide arginine under normal conditions. Hepatic release of urea cycle intermediates is also not known to occur (Windmueller and Spaeth 1981) because there is a tight channeling of urea cycle intermediates within the liver (Cheung et al. 1989). To the authors' knowledge, the hepatic zonation of arginase and other urea cycle enzymes has not been examined in the neonate, however, and this may affect the release of arginine and other urea cycle intermediates from the neonatal liver. In summary, although the arginine biosynthetic enzymes are abundant and highly active in the liver, the high activity of hepatic arginase, coupled with the tight channeling of urea cycle intermediates, precludes a role for the liver in regulating whole body arginine homeostasis under normal conditions.

Urea cycle enzymes are also present in the kidney, the major site of arginine synthesis in the adult. Although the adult kidney lacks CPS-1 and ornithine transcarbamylase (OTC), argininosuccinate synthase (ASS) and ASL are present in the proximal tubules (Morris 1992), and thus intestinally derived citrulline is converted to arginine. Renal arginase activity is low relative to liver (Edmonds et al. 1987b), and differs from the hepatic form. This isoform is predominantly biosynthetic, with ornithine as its most important product and urea synthesized only incidentally (Iyer et al. 1998). Furthermore, because the majority of renal arginase activity is present in the cortex, and medullary blood flow bypasses this area, arginine synthesized in the medulla can be released into the general circulation (Edmonds et al. 1987b). There is, however, an important distinction between renal arginine synthesis in the neonate and the adult. Whereas the activity of ASS and ASL is low in the intestine and high in the kidney of the

adult (Morris 1992), this situation is reversed in the neonate (Wu and Knabe 1995a), thereby limiting renal arginine synthesis in the neonate.

Although these data suggest that the liver and the kidney are not involved in neonatal arginine synthesis under normal conditions, urea cycle enzyme and cationic amino acid transporter activities may be altered under abnormal dietary conditions. There is evidence that factors such as starvation, high protein feeding, and glucagon treatment can increase the hepatic activities and mRNA levels of all urea cycle enzymes (Morris 1992). Hepatic ornithine aminotransferase (OAT) activity, for instance, is induced in rat liver under conditions of high protein feeding (O'Sullivan et al. 2000), as is the activity of the hepatic y^+ transporter. High protein diets are associated with elevated glucagon levels, the administration of which stimulates y^+ transporter activity in isolated rat hepatocytes (Handlogten and Kilberg 1984). Because an increased dietary supply of arginine and glutamine has an effect similar to glucagon in both rat and human hepatocytes (Espat 1996), dietary arginine, via glucagon, may regulate the activity of the y^+ transporter in hepatocytes. Overall, glucagon plays a significant role in the regulation of hepatic amino acid metabolism (O'Sullivan et al. 2000). Chronic glucagon administration coordinately induces the activity of all 5 urea cycle enzymes (Ulbright and Snodgrass 1993), and specifically enhances hepatic arginase activity (O'Sullivan et al. 2000). The ability of dietary arginine to regulate endogenous arginine synthesis may therefore be mediated via its influence on glucagon secretion. Arginine is in fact a secretagogue for a number of hormones including insulin, growth hormone and prolactin, and therefore the potential for these and other hormones such as cortisol (Flynn and Wu 1997) to influence urea cycle enzyme activity may be important. Finally, in the rat

kidney, high protein feeding causes a doubling in the activity of "arginine synthetase" (ASS + ASL) (Szepesi et al. 1970) and in the relative abundance of mRNA for the enzymes of arginine synthesis (Morris et al. 1989). Such a diet also increases renal plasma flow, thereby elevating renal citrulline uptake and the corresponding renal arginine release (Dhanakoti et al. 1992).

In addition to the possibility of arginine synthesis by the liver or kidney under abnormal dietary circumstances, a number of urea cycle enzymes have extrarenal and extrahepatic locations (Ratner 1973, Windmueller and Spaeth 1981). Windmueller and Spaeth (1981) observed net citrulline release from the skeletal muscle in the rat, and also found evidence of extrarenal and extrahepatic citrulline utilization. Whether or not these other tissues can make a significant contribution to arginine synthesis is not known.

The fractional net conversions of proline to citrulline and ornithine support the observations we have made from the arginine data. In adequately fed pigs, the gut, operating at its basal and maximal level, converted more proline to citrulline and ornithine on first pass than during IP infusions. When dietary arginine was in short supply, deficient animals upregulated the conversion of proline to citrulline and ornithine, presumably via one of the previously described mechanisms, to match the level of first pass gut conversion. Across diet levels, the fractional net conversion of proline to ornithine generally followed the pattern observed for arginine in Table 4.3.4 (fractional net conversions are presented as % of arginine flux), although the first pass intestinal conversion of proline to ornithine was significantly greater in deficient animals, which was not true for the conversion of proline to arginine. Ornithine flux was not measured in this study, however, and a decline in ornithine flux during feeding of an arginine

deficient diet, similar to what Castillo et al. (1994b) observed, may alter the magnitude of the differences observed in the fractional net conversion rates of proline to ornithine. Fractional net conversions of proline to citrulline did not follow the same pattern as ornithine, however Castillo et al. (1993a, 1994b, 1995b) have previously shown in adults that the flux of ornithine, but not of citrulline, mimics the decline in arginine flux during feeding of an arginine free diet. Ornithine may therefore be more sensitive to dietary arginine perturbations than citrulline, as our plasma amino acid data also suggests.

Although the turnover time of the urea pool is very slow in the adult at maintenance (Wolfe 1992), it is much faster in young growing animals. We have previously demonstrated that the half-life of urea in the neonatal piglet is approximately 3.5 hours, with a turnover time of 5 hours (Bertolo PhD Thesis 1999). Consequently, a plateau in the isotopic enrichment of plasma can be achieved much more easily and quickly in the neonate than in the adult. Indeed, we were able to reach plateau in urea near the end of a 6.5 hour L-[guanido-^{14}C]arginine infusion. The conversion of arginine to urea was estimated from the SRA data at plateau.

Although there is little exchange between hepatic and plasma arginine pools in adults (Castillo et al. 1994a), there are important differences between arginine metabolism in the neonate and the adult, and the compartmentation of these pools has not been examined in the neonate. In this study, it appears that the arginine status of the 2 groups of animals was sufficiently different that changes in urea synthesis from arginine were evident in the plasma compartment. We did not measure the contribution of the gut to urea synthesis, although this value was likely very low because arginase activity is negligible in neonatal piglet enterocytes (Wu 1995b). It is clear from our BUN data that

the urea pool was larger in animals fed the arginine deficient diet, and thus urea flux was likely higher in these animals. However, because we did not quantify urea flux, we cannot conclude from these data that the net conversion of arginine to urea is significantly reduced during feeding of an arginine deficient diet. Indeed, BUN data suggests that this conversion was higher during arginine deficiency.

Data generated from primed, constant infusions of glutamate were difficult to translate into meaningful kinetic data. The isolation of the radioactivity associated with glutamate was complicated by the close proximity of aspartate and glutamate in our HPLC elution profile, and consequently, precise separation of the peaks was not possible. Furthermore, it was difficult to distinguish between ^{3}H arising from the internal ^{3}H leucine standard, from the ^{3}H associated with the amino acids of interest, thus affecting the calculation of plateau SRA values. It is perhaps for this reason that values for glutamate flux did not reflect the substantial extraction of dietary glutamate that was shown to occur on first pass by the gut in 28 day old piglets (Reeds et al. 1996). This phenomenon has not, however, been studied in neonatal piglets. Finally, subsequent analysis demonstrated that the glutamate tracer was only 79% pure. The recovery of ^{3}H in arginine was very small, however, and therefore a 21% increase in the recovery of radioactive label in arginine would not alter our conclusions, because the radioactivity in the tritium channel of arginine would only increase to a maximum of 24 dpm.

Despite the difficulties encountered in efforts to quantify $Q_{glu \rightarrow arg}$, these results have demonstrated that $Q_{glu \rightarrow arg}$ is biologically insignificant for the neonate. This conclusion concurs with Murphy et al's (1996) findings in neonatal piglets, and with the work of Wu et al (1994) and Blachier et al (1993) in neonatal piglet enterocytes. As

described in Chapter 3.0, short term infusion studies of glutamate metabolism are complicated by its large, widely distributed pools (Reeds et al. 2000), and by the marked intra and extracellular compartmentation of glutamate metabolism (Reeds et al. 1996, 1997). As such, glutamate kinetics may only reflect transport rates through plasma as opposed to whole body fluxes (Darmaun et al. 1986). Although glutamate kinetic data may not be entirely predictive of true intracellular events, enzyme activities measured by Wu et al (1994) independently support the kinetic data generated in the present investigation. Furthermore, piglets fed a deficient level of dietary arginine had 0.88g/kg/d of available arginine from a combination of diet and intestinal synthesis from proline, however, these animals still experienced episodes of hyperammonemia, a phenomenon that would not have occurred if they were synthesizing significant quantities of arginine from glutamate. Because of the marked changes in the activities of the enzymes that synthesize arginine from glutamate/glutamine during the course of early development, our conclusions regarding the suitability of glutamate as a precursor for arginine synthesis are age specific, and apply only to a 6 to 9 day old piglet.

IG arginine fluxes were used in this study for calculations of intestinal arginine synthesis. Because arginine is synthesized in the gut of neonates, it did not seem reasonable to exclude the effects of the gut from our conversion estimates. IG flux represents whole body flux, and regardless of whether IV, IP, or IG fluxes are used for calculation purposes, the relative differences between diets in $Q_{pro \rightarrow arg}$ are real. The major findings of this research confirm the existence of a basal and a maximal rate of arginine synthesis, and of dietary regulation of arginine synthesis from proline. These findings are independent of specific flux values.

It is not clear from this study whether plasma NO concentrations are significantly impacted by the level of arginine provided in the diet. There was a trend suggestive of impaired NO synthesis in arginine deficient animals, which was similar to the reduction in NO synthesis by cNOS and iNOS observed by Wu et al (1999) in arginine deficient rats, although the magnitude of the response was smaller in the rats. To the author's knowledge, no other comparable studies of the influence of dietary arginine on NO synthesis exist in healthy neonates. Although there were no statistically significant differences in NO production between diets, the magnitude of the observed differences is sufficient to exert a biological impact, as evidenced by the ability of iNOS stimulation to substantially change the endogenous action of NO. That dietary arginine deficiency may impact NO production is of extreme importance to the health of neonates, because decreased NO production is associated with neonatal disease such as PPHN and NEC. Indeed, plasma arginine concentrations are lower in infants affected with these conditions (Vosatka et al. 1994, Zamora et al. 1997, Pearson et al. 2001, Amin et al. 2002), and arginine supplementation can significantly reduce the incidence of NEC (Amin et al. 2002). Conversely, overproduction of NO can contribute to tissue injury, hypotension, and myocardial failure (Bruins et al. 2002).

The turnover time of the proline pool is affected by the dietary concentration of arginine, but not by the choice of proline label (2,3-^{3}H vs U-^{14}C). Thus, the differences observed between the P/F ratios derived for proline in this study compared to Bertolo's work (PhD Thesis 1999), were caused by differences in the dietary arginine concentration. The P/F ratio is equal to the turnover time, and represents the time required for the total pool to move in and out of the system (Shipley and Clarke 1972).

The ratio in this study (2.0) was twice the value determined by Bertolo (1.0) (PhD Thesis 1999), indicating that the arginine deficient and adequate diets we administered caused the proline pool to turn over more slowly compared to feeding an intermediate quantity of arginine. This may have been caused by the 1.5 to 2 fold greater proline pool size of our pigs compared to Bertolo's (PhD Thesis 1999). Because plasma proline concentrations were not different between diets, this expansion must reflect the intracellular proline pool size. In arginine adequate pigs less proline was required for conversion to arginine, thus increasing the pool size of proline. In arginine deficient pigs, greater protein breakdown, or a reduction in protein synthesis, may have increased the size of the proline pool.

In conclusion, we have demonstrated that the arginine synthetic capacity of the neonatal piglet is limited to a specific range, with both basal and maximal levels. When arginine is deficient in the diet, whole body arginine synthesis is capable of doubling, in an attempt to meet the increased demand. This synthetic ability confers a certain level of flexibility during enteral arginine administration to the healthy neonate, however it also highlights the need to provide supplemental arginine in conditions that increase the metabolic demand for arginine. First pass arginine synthesis from proline by the gut appears to operate at its maximal capacity independent of the dietary intake of arginine. When the supply of arginine is limited in the diet, the conversion of proline to arginine is upregulated by some means other than first pass intestinal contribution, so that the two contribute equally to arginine synthesis. Because proline is the major precursor for such synthesis, it must be supplied in the diet, however glutamate does not appear to be a significant precursor for arginine synthesis in this model.

5.0 GENERAL SUMMARY AND FUTURE DIRECTIONS

The overall objective of this research was to determine whether endogenous arginine synthesis is under dietary regulation in the neonatal piglet. Although dietary arginine does not appear to regulate arginine synthesis in the adult (Castillo et al. 1993a), arginine is dispensable for healthy adults, whereas it is indispensable in the diet of preterm neonates (Brunton et al. 1999). Based on this important difference, it seems logical to predict that the availability of dietary arginine for the neonate may play a significant role in determining the level of metabolic activity directed toward arginine synthesis, particularly because energy intake is a limiting factor for these infants (Groh-Wargo et al. 1994). By feeding what we determined to be an adequate and a minimal dietary inclusion level for arginine, we have described the existence of a basal level of arginine synthesis that occurs even when there is an adequate supply of arginine. We have also documented a maximal level of arginine synthesis that was not sufficient to satisfy the metabolic demand for arginine when dietary arginine was provided at a minimal inclusion level. These data have furthermore demonstrated that dietary arginine is an important regulator of endogenous arginine synthesis in the neonatal piglet, and that proline, but not glutamate is an important precursor for neonatal arginine synthesis.

We have previously demonstrated that small intestinal, and in particular, first pass gut metabolism is essential for the conversion of proline to arginine in the neonate (Bertolo et al. 2002). In support of this result, first pass small intestinal $Q_{pro \rightarrow arg}$ accounted for 42% of the total $Q_{pro \rightarrow arg}$ in animals fed a deficient level of arginine, and 64% of the total $Q_{pro \rightarrow arg}$ in animals fed an arginine adequate diet. The higher percentage in adequately fed animals results from the maintenance of basal, but maximal, first pass

intestinal conversion of proline to arginine, combined with lower overall whole body arginine synthesis from proline. Although we previously concluded that the conversion of proline to arginine was negligible during IP infusions, these observations were indirect, because the fractional net conversions of proline to ornithine, and of ornithine to arginine were quantified separately (Bertolo et al. 2002). Furthermore, arginine synthesis in that study was quantified under very different dietary conditions, because arginine was provided at 1.00g/kg/d. It is important to emphasize that all of our current and previous data represent *net* conversions, which are clearly dependent upon our ability to detect in the plasma, the products of specific intracellular conversions of precursor to product. As such, these values represent *minimum net* conversions of precursor to product, of which some may be more readily detectable in the plasma than others. That there was conversion of proline to urea cycle intermediates during IP infusions in our previous work is evident from the net fractional conversion of proline to citrulline of 17% (Bertolo PhD Thesis 1999). These papers are therefore complementary in that they supplement the information provided by the other, and contribute different pieces to the arginine puzzle.

Our data enables speculation regarding the optimal quantity of arginine to provide for the preterm neonate. On a whole body scale, piglets receiving 11% of their metabolic requirement for arginine via the diet were able to synthesize an additional 38% of this requirement from proline. Animals provided with 100% of our estimate of the metabolic arginine requirement synthesized an additional 20% of this requirement from proline. Clearly, during TPN feeding, the entire metabolic requirement for arginine must be satisfied through the diet. During enteral feeding however, these data demonstrate that

there is a minimum of 0.26g/kg/d of arginine that will be synthesized on first pass by the gut, and a minimum of 0.13g/kg/d that will be synthesized via some other means. Therefore, if 0.39g/kg/d of arginine synthesis from proline is obligatory, it is proposed that delivering 1.41g/kg/d of enteral arginine will exactly satisfy the metabolic requirement. This speculation assumes that proline is provided at a level that satisfies the whole body proline requirement, with additional substrate available for conversion to arginine. Furthermore, if non-first pass arginine synthesis from proline is upregulated to a maximum of 0.40g/kg/d, whole body arginine synthesis from proline can maximally provide 0.66g/kg/d. A minimum of 1.14g/kg/d of arginine must therefore be provided in enteral diets to satisfy the metabolic arginine requirement. This estimate is supported by the demonstration that muscle protein synthesis, plasma arginine, and plasma ammonia concentrations plateau when arginine is provided at intakes between 1.20 and 2.00g/kg/d (Wilkinson et al. 2002). Such speculation is not meant to define specific parameters for dietary arginine feeding, but rather to illustrate how data of this type can be used in the clinical setting to optimize the delivery of arginine to preterm infants, whose immaturity or health status may limit protein tolerance.

Swine and human milk do not contain sufficient quantities of arginine. Indeed, the administration of human milk to the piglet would provide only 0.54g/kg/d of arginine (based on a protein intake of 15g/kg/d), which is only slightly lower than the arginine intake from sow's milk (0.66g/kg/d) (Pencharz et al. 1996). Consequently, human milk can only supply half of the daily arginine requirement for protein deposition in a newborn infant. Human milk is, however, intended for intake by term infants, who have the distinct advantage of a healthy, and relatively mature gut compared to TPN fed preterm

infants. Therefore, the abundance of proline in milk (Davis et al. 1994) probably contributes to endogenous arginine synthesis in milk-fed term neonates.

The range of arginine concentrations in commercial TPN solutions is from 4.7-12.3g/100g amino acids (Brunton et al. 2000). This corresponds to feeding a neonatal piglet 0.71 to 1.85g/kg/d of arginine, which is adequate only at the highest arginine concentration. During TPN feeding, gut atrophy and the lack of first pass intraluminal precursors preclude arginine synthesis, and therefore the entire metabolic requirement for arginine must be satisfied through the diet. In this study, piglets enterally fed a deficient level of arginine (0.20g/kg/d) had 0.88g/kg/d of arginine (diet + synthesis) available for metabolism. Although these piglets did not demonstrate any overt symptoms of arginine deficiency, metabolically they exhibited elevated plasma urea and ammonia levels, low plasma arginine concentrations, and a trend toward lower NO levels compared to adequately fed pigs. Indeed, muscle protein synthesis and plasma ammonia concentrations did not reach optimal levels in TPN fed piglets when arginine was provided below 1.20g/kg/d (Brunton et al. 2001). These data clearly show that intravenous administration of low arginine TPN solutions will precipitate arginine deficiency. This condition may not be readily apparent to health care providers because of the absence of overt deficiency symptoms.

In this thesis, piglets fed arginine deficient diets exhibited high plasma glutamine levels. These elevated levels resulted from the need to detoxify excess ammonia that could not be processed by the urea cycle, and may have important implications for TPN fed infants. Glutamine is not included in TPN solutions because of concerns about its stability (Pencharz et al. 1996), although some advocate for its inclusion (Furst 2001).

Because most TPN formulations do not contain sufficient arginine, the addition of glutamine can further increase the need to dispose of ammonia from glutamine oxidation (Bertolo et al. 1999b). Furthermore, inclusion of glutamine, without concomitant adjustment of the arginine concentration, may overwhelm the capacity for urea synthesis in neonates, leading to hyperammonemia.

There was a strong trend towards a reduction in NO synthesis when piglets were fed insufficient quantities of dietary arginine. This effect of arginine deficiency on NO synthesis is of particular significance, in light of recent research demonstrating that plasma arginine concentrations are lower in preterm infants who develop NEC than in those who do not, and that arginine supplementation significantly lowers the incidence of NEC in this population (Amin et al. 2002). If dietary arginine deficiency can indeed impair NO synthesis in a healthy neonate, the reformulation of TPN solutions to include more arginine may have a substantial impact on neonatal health and survival.

A number of avenues for future research emerge from our data. Because a reduction in the rate of arginine degradation functions to conserve arginine in adults fed arginine free diets (Castillo et al. 1994b), it would be useful to determine whether this mechanism also operates in the neonate. The question of how arginine synthesis is upregulated in enterally fed animals, either via arterial amino acid uptake, or by the liver, kidney, or other tissues, is also an intriguing one. If arterial amino acid uptake by the intestine proves to be the mechanism, this result would provide further support for the practice of minimal enteral feeding. Along with this question, the ability of the TPN fed neonate to convert proline to arginine by these same means should be investigated, particularly because there is a plateau in many indices of arginine adequacy when

arginine is provided between 1.20 and 2.00g/kg/d (Brunton et al. 2001, Wilkinson et al. 2002). NO synthesis in healthy neonates deserves special attention, and should be investigated with the more sensitive techniques of HPLC analysis (Lagerwerf et al. 1998). Of prime importance, however, is the elucidation of the specific arginine requirement of the neonate, and the adjustment of TPN formulations to coincide with the true neonatal arginine requirement.

6.0 LITERATURE CITED

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